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SOME EFFECTS OF CYANATE ON PHOSPHORYLASES *a* AND *b*

by

CHIN-CHIANG HUANG

A THESIS
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "SOME EFFECTS OF CYANATE ON PHOSPHORYLASES a AND b", submitted by CHIN-CHIANG HUANG in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Phosphorylases a and b have been found to be inactivated by potassium cyanate. The rate of inactivation is paralleled by the rate of incorporation of cyanate into the protein, and both processes follow pseudo-first order kinetics. The nature of the kinetics has been investigated. The reaction of cyanate with phosphorylase has been shown to involve the carbamylation of the ε-amino groups of lysine to form homocitrulline residues. Sulfhydryl groups do not react, nor is pyridoxal-5'-phosphate displaced from the protein. Although glucose-1-phosphate and adenosine-5'-phosphate decrease the rate of inactivation by cyanate, there is as yet no evidence that the ε-amino groups of lysine play a direct role in the catalytic process of this enzyme. The carbamylation of phosphorylase a is accompanied by a dissociation of the protein into halves.

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LIST OF ABBREVIATIONS

AMP	adenosine-monophosphate
ADP	adenosine-diphosphate
ATP	adenosine-triphosphate
ATEE	acetyltyrosine ethyl ester
$E^{1\%}_{cm}$	extinction coefficient of 1% protein
FDNB	fluorodinitrobenzene
G-1-P	glucose-1-phosphate
K_m	Michaelis constant
K'	first order rate constant
PCA	perchloric acid
PCMB	p-chloromercuribenzoate
Pi	inorganic phosphate
PLP	pyridoxal-phosphate
S	sedimentation coefficient
$S^{o}_{20,w}$	sedimentation coefficient in water at 20°C at infinite dilution
TCA	trichloroacetic acid
V_{max}	maximum velocity
Versene	disodium ethylene diaminetetraacetate
RTIC	rotor temperature indicator and control

I. INTRODUCTION

Phosphorylase (EC 2.4.1.1.α-1,4,Glucan; orthophosphate glycosyltransferase) is the enzyme which catalyzes the synthesis and degradation of the α-1,4 bonds of glycogen in the reaction:

$$\text{Glycogen} + \text{Glucose-1-Phosphate} \rightleftarrows \text{Glycogen-Glucose} + \text{Phosphate}$$

The reaction occurs in most mammalian tissues, yeast (1), plants (2), and constitutes the first step in the utilization of glycogen by the organism (1,3,4). The reaction was shown to be reversible (5). In muscle extracts this reaction was elucidated chiefly by the Cori's and their coworkers (4-8). The behavior of the enzyme of the dialyzed muscle extracts showed minimal phosphorylase activity unless catalytic amounts of adenosine-5'-phosphate were added (9). In contrast, the yeast and potato phosphorylases are not activated by adenosine-5'-phosphate. Furthermore, the muscle enzyme was found to require a small amount of glycogen as a "primer" in the synthetic direction (7); thus the enzyme merely adds or removes glucose residues at the terminal of a branched polysaccharide chain.

In 1942 the preparation of crystalline phosphorylase was announced by Green and Cori (10). The crystallized enzyme showed 60-70% of its maximum activity in the absence of AMP (11). In fact it was found that there were two forms

of phosphorylase: the crystalline enzyme, having the activity characteristic mentioned, and a second, more soluble form, having an absolute requirement for AMP. The former was designated "phosphorylase *a*" and the latter "phosphorylase *b*" (11). In the presence of AMP the two enzymes were said to be equally active.

The two forms of glycogen phosphorylase in rabbit muscle were found to be interconvertible under certain conditions. The present concept of the conversion of phosphorylase *b* to phosphorylase *a* maintains that the former is phosphorylated and dimerized by the action of a specific phosphorylase kinase which requires ATP and Mg^{++} (12). It is now known that four moles of phosphate are incorporated into the enzyme molecule at the site of serine residues (13). The reverse reaction is catalyzed by an equally specific phosphatase (formally called PR enzyme) (14,15), and by trypsin at pH 6.0 (13). Although the product of the latter cannot be reconverted to phosphorylase *a* by phosphorylase kinase, the activity and molecular weight is similar to phosphorylase *b*. The reactions can be written as follows:

$$2 \text{ phosphorylase } \underline{b} + 4 \text{ ATP} \xrightarrow[\text{Mg}^{++}]{\substack{\text{phosphorylase} \\ \text{kinase}}} \text{phosphorylase } \underline{a} + 4 \text{ ADP}$$

$$\text{Phosphorylase } \underline{a} \xrightarrow{\substack{\text{phosphorylase} \\ \text{phosphatase}}} 2 \text{ phosphorylase } \underline{b} + 4 \text{ Pi}$$

$$\text{Phosphorylase } \underline{a} \xrightarrow[\text{pH } 6.0]{\text{trypsin}} 2 \text{ phosphorylase } \underline{b}' + 4 \text{ phosphate peptide.}$$

The molecular weight of phosphorylase a is 495,000 ($S^{o}_{20,w}$ = 13.2S) while that of phosphorylase b is one half as large; 242,000 ($S^{o}_{20,w}$ = 8.2S) (15). Madsen and Cori (16) discovered in 1955 that modification of the cysteine residues of phosphorylase a by reaction with P-chloromercuribenzoate resulted in a loss of enzymatic activity and a concurrent dissociation into subunits of molecular weight 125,000 ($S^{o}_{20,w}$ = 5.6S). Similarly, phosphorylase b was inactivated and reduced to one half of its size. These effects of the mercurial could be reversed by cysteine. This data appears to indicate that phosphorylase b is a dimer and phosphorylase a is a tetramer of a subunit or protomer.

Recently, another parameter has been added to the phosphorylase structure. Baranowski et al (18) have found that there are four moles of pyridoxal-phosphate bound to phosphorylase a and two moles bound to phosphorylase b. The point of attachment is from the carbonyl group of pyridoxal-5-phosphate to the amino group of a lysyl residue. It has been shown that upon removal of this coenzyme from phosphorylase there is a loss not only of activity but also of structural integrity (19). Both effects are reversible by the readdition of PLP. Fischer et al (20) have found no loss of activity when PLP is covalently bound to the enzyme by $NaBH_4$ reduction. The PLP coenzyme has been found in phosphorylase from human muscle (21), rabbit heart muscle (22), lobster muscle (23) and potatoes (24). It was found that in high ionic strength (2.5 M NaCl) at pH 7.4 the enzyme dissociated into a dimeric form of $S^{o}_{20,w}$ = 8.3S (25), but

no monomer formed at this salt concentration. At lower pH's (6.8) PLP was also lost from the protein. This suggested that the quaternary structure of phosphorylase a is stabilized by electrostatic forces and the types of interaction between the individual monomeric units of phosphorylase a are not identical. Furthermore, Wang and Graves (56,57) also found that the dimeric form of phosphorylase a was observed in 0.05 M glucose solution, or at lower protein concentration and this dissociation is favored by preincubation of the enzyme with AMP. In addition to a study of the relationship of the dimeric structure of phosphorylase a they found that preincubating the enzyme in glucose resulted in approximately a three-fold increase in enzymatic activity. The same result was obtained upon dilution of the enzyme concentration. On the basis of these kinetic and ultracentrifugal studies, they suggested that the dimeric form of phosphorylase a is more active than the tetrameric species, and the appearance of enzymatic activity that occurs in the conversion of phosphorylase b to a is more directly related to the phosphorylation of the protein than to molecular alteration and that dimerization of the protein is only a secondary effect.

In 1956 Hartley and Massey (45) studied the active center of chymotrypsin by reacting it with fluorodinitrobenzene (FDNB). Under the mild conditions of low temperature (-3°C) in the presence of 17% ethanol they found that only 6 of 13 lysine residues would react with FDNB and that 3 moles of tyrosine also reacted. The K_m of the substrate

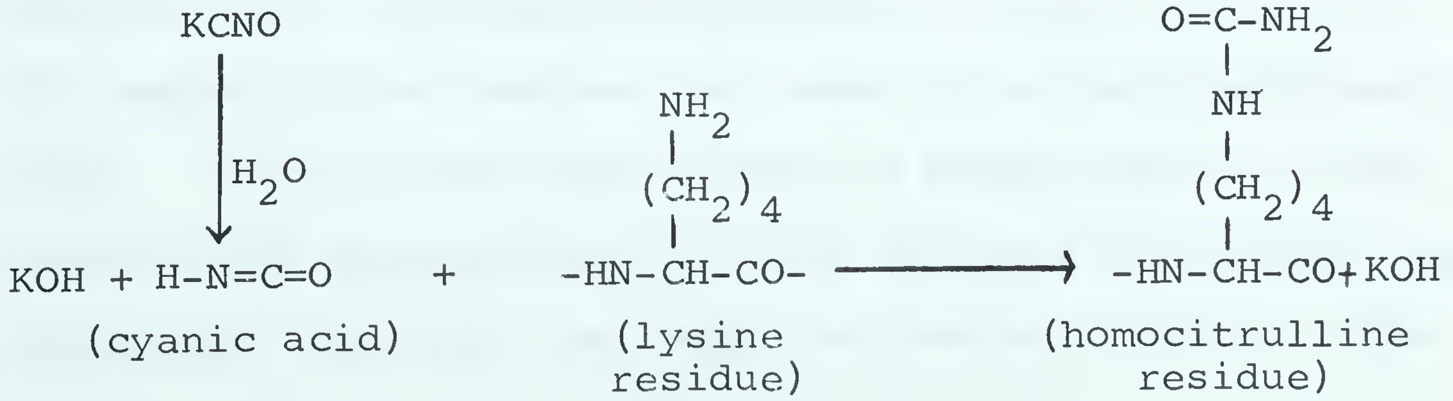
acetyltyrosine ethyl ester (ATEE) increased with increasing time of reaction with FDNB while the maximum velocity remained unchanged. This suggested that -lysine groups or tyrosine hydroxyl groups are required in the formation of the enzyme substrate complex, but not involved in the catalytic activity of the enzyme. This finding that lysine residues may play a role in the formation of the enzyme substrate complex may be true for other enzymes also.

Hopkins and Wormall (46,47,48) and Fraenkel-Conrat (49) have shown that isocyanate reacts readily with amino groups of protein in aqueous solutions of pH 8.0 at 0°C. In their studies they used phenylisocyanate and p-bromophenylisocyanate (46) on such proteins as casein, horse serum albumin (47) and insulin (48). They found in general that good agreement was obtained between the loss in amino groups and the number of p-bromophenylisocyanate residues introduced as calculated from the bromine analyses. They also obtained complete inhibition of serological reaction between phenylisocyanate treated horse serum albumin and its antiserum.

In 1960 Stark *et al* (36) showed that ribonuclease not only lost enzymatic activity but also exhibited an altered amino acid content when this enzyme was incubated with 8 *M* urea at 40° for several hours then dialyzed to remove the urea. In their amino acid analysis of this 8 *M* urea treated ribonuclease on IRC-50 chromatography they found the lysine content was less than that of ribonuclease *a*, and in addition, a new peak appeared on the effluent curve from the

ion exchange column just in front of and partly overlapping the position of valine. However, they were able to separate this new peak with Amberlite IR-120 with a change in the operating temperature from 30° to 50° at 280 ml. This new amino acid was identified as homocitrulline by a comparison with a standard mixture of amino acids in the system employed by Spackman *et al* (34). The amount of this new amino acid increased and the amount of lysine concomitantly decreased as the time during which ribonuclease was exposed to urea was lengthened. The decrease in enzymatic activity and the formation of homocitrulline were much more rapid when the ribonuclease was incubated with 1 *M* KCNO at pH 8.1 at 50°C for one hour. It was evident that a chemical reaction involving the lysine residues of ribonuclease was taking place in urea solution. They suggested that the observed loss of lysine had come about as a result of carbamylation of the ε-amino group by cyanate in the urea solution. The studies of Dirnhuber and Schütz (55) indicated that at equilibrium an 8 *M* urea solution at pH's more alkaline than about 6.0 would be 0.02 *M* in respect to cyanate. In view of these observations in the literature and the effect noted with ribonuclease, they concluded that cyanate does react with amino and sulfhydryl groups in proteins.

The reaction between KCNO and the lysine group may be represented as follows:



The work reported in this thesis has been directed to determine whether some lysine residues of phosphorylase were important for the enzymatic activity and the stabilization of the protein structure. The effect of KCNO on phosphorylase was studied from several aspects: 1) a kinetic study of the inactivation of phosphorylase by KCNO; 2) a study of the incorporation of $K^{14}CNO$ into phosphorylase; 3) pyridoxyl-5-phosphate content of carbamylated enzyme; 4) amino acid composition of carbamylated phosphorylase; 5) ultracentrifugal studies of carbamylated phosphorylase; 6) disc electrophoresis behavior of carbamylated phosphorylase compared with PCMB treated phosphorylase.

The results showed that both phosphorylases a and b lost enzymatic activity upon carbamylation by KCNO. The rate of inactivation followed an apparent first order type of kinetics. Furthermore the carbamylation of these enzymes was accompanied by dissociation of the enzyme molecule. Since the enzymatic inactivation is much faster than the dissociation of the enzyme molecules, the dissociation may be a secondary effect of the carbamylation of lysine groups. The carbamylated phosphorylases also reveal a homocitrulline peak upon amino acid analysis of these enzymes with an amino acid analyzer (Beckman model 120B). The prosthetic group of phosphorylase, pyridoxal-5-phosphate, was not involved in the carbamylation reaction, as shown by a spectrophotometric study. It was found that at least 9 bands appear in both carbamylated phosphorylase a and b analyzed with a disc electrophoresis technique and only one band was found in PCMB

treated enzymes. As shown by ultracentrifugal studies, the dissociated enzymes are of only two types, dimer and monomer. Thus the heterogeneous bands shown by a disc electrophoresis technique are a kind of charge heterogeneity. The lysine residues in the phosphorylases may play the role of binding sites or auxilliary binding sites as well as in maintaining the integrity of the natural configuration of those enzymes through electrostatic forces. Thus the carbamylation of the lysine residues results in the loss of enzymatic activity and dissociation of these enzymes.

II. MATERIALS AND METHODS

Crystalline phosphorylase b was prepared from rabbit muscle as described by Fischer and Krebs (26) and crystalline phosphorylase a was prepared from rabbit muscle by the method of Green and Cori (27) or from phosphorylase b by the method of Krebs, Kent and Fischer (28). These enzymes were recrystallized four or five times, treated with acid washed activated charcoal (Norit A) and passed through a Sephadex G-25 gel filtration column in order to remove AMP, Mg^{++} and other impurities. The latter steps were carried out as follows.

The crystalline suspension of phosphorylase a or b was centrifuged at 0°C in a Servall RC-2 automatic refrigerated centrifuge at 10,000 r.p.m. for 10 minutes and the supernatant fluid was discarded. The crystals were dissolved in 0.02 M sodium glycerophosphate - 0.0015 M Versene buffer at pH 6.8 at 30°C for phosphorylase b and 35°C for phosphorylase a. A small amount of water and buffer washed Norit A was added to the enzyme solution and the mixture was stirred at room temperature for about 5 minutes. After centrifugation, also at room temperature at 10,000 r.p.m. for 10 minutes, the supernatant was filtered through acid washed filter paper, then passed through a column of Sephadex G-25 gel which had been pretreated with the same buffer solution. The eluted fractions were collected and examined at 280 mμ and 260 mμ in a spectrophotometer.

The elution pattern is shown in Figure 1 (this protein had not been treated with Norit A). It may be seen that the protein is completely separate from the nucleotide fraction (AMP). A qualitative colorimetric test indicated that the first nucleotide peak contained magnesium.

Phosphorylase b kinase was prepared from rabbit muscle by the method of Krebs and Fischer (29).

Ammonium sulfate was a Mann Research Laboratories, Inc. preparation and a saturated solution was boiled with activated charcoal (Norit A) and neutralized with ammonium hydroxide to pH 6.8. Glucose-1-phosphate dipotassium ester was also obtained from Mann Research Laboratories, Inc. Glycogen (rabbit liver) was purchased from the same company and purified by passing through a Dowex-1 chloride column and neutralized. Adenosine-5'-phosphate (AMP) and parachloromercuric benzoate (PCMB) were obtained from Sigma Chemical Company. Sephadex G-25 for gel filtration was a product of Pharmacia, Uppsala, Sweden. KCNO was bought from Fisher Scientific Company and used without further purification. Sufficient KCNO to make a 1.0 M solution was weighed into a 10 ml volumetric flask and dissolved in 0.1 M glycerophosphate - 0.0015 M Versene buffer, pH 6.8, just before use. $K^{14}CNO$ (7 mc/mM) was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. According to the method of Bader et al (30), the $K^{14}CNO$ was dissolved in distilled water and kept frozen until use. The other chemicals were all reagent grade and were used without further purification.

Phosphorylase activity was measured in the direction

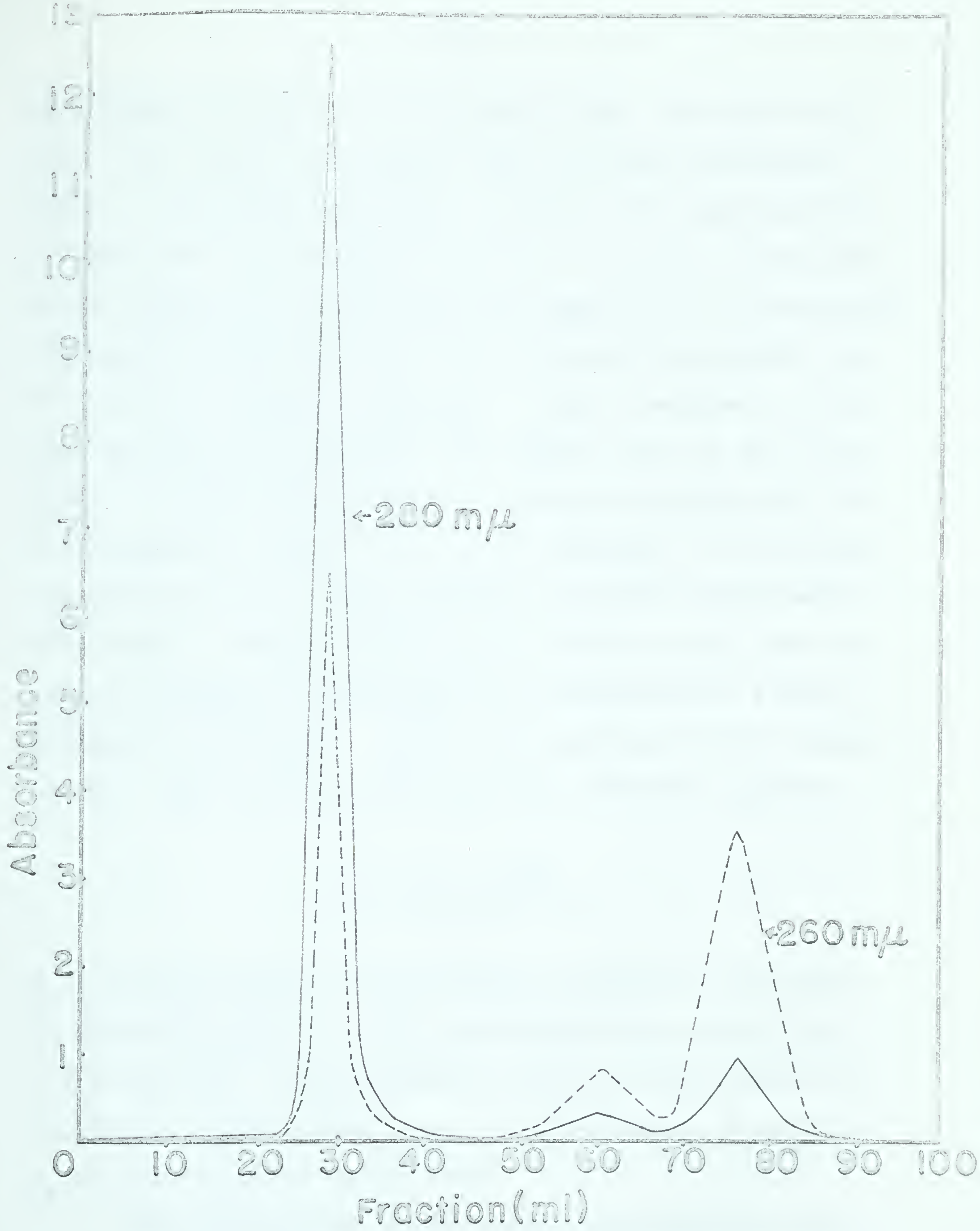


Figure 1. Sephadex G-25 chromatography of phosphorylase b.
Solid line (———) optical density at 280 mμ
Broken line (----) optical density at 260 mμ
G-25 column, (2.2 x 17 cm) Buffer solution, 0.02 M
Na-glycerophosphate - 0.0015 M Versene, pH 6.8

of glycogen synthesis as described by Cori, Cori and Green (31). The enzyme was diluted with 0.02 M Na glycerophosphate - 0.0015 M Versene buffer, pH 6.8, to a concentration of about 40-60 μg of protein per ml. To 0.1 ml of this diluted solution in a Klett tube was added 0.1 ml of substrate containing 2% glycogen and 0.032 M glycose-1-phosphate, pH 6.8, with or without 0.002 M AMP. After 5 minutes at 30°C, the reaction was stopped by the addition of 7 ml of 0.07 M H_2SO_4. In the determination of inorganic phosphate by the Fiske-Subbarow method (32), 0.9 ml 5 N H_2SO_4, 1 ml 2.5% NH_4 molybdate and 0.5 ml amino-naphthol sulfionic acid reagent were added. Following a 10 minute waiting period, the inorganic phosphate concentration was determined on a Klett Summerson colorimeter. Units were calculated by the method of Cori, Cori and Green (31) from the following equation:

$$K = \frac{1}{t} \log_{10} \frac{X_e}{X_e - X} \qquad [1]$$

X_e is the percentage of the glucose-1-phosphate converted at equilibrium and X is the percentage converted at time t (in minutes). K multiplied by 1,000 has been defined as "units" of enzyme activity per ml of reaction. At pH 6.8, X_e has the value of 78 per cent.

The phosphorylase concentration was determined with a Zeiss PMQ II spectrophotometer from the absorbance at 280 mμ using a value for the $E_{cm}^{1\%}$ of 11.8, calculated from the data of Velick and Wicks (33).

The $K^{14}CNO$ content of phosphorylase was determined

by precipitating the reaction mixture with cold 5% TCA and washing the protein with 5% TCA during millipore filtration. The denatured enzyme protein on the millipore filter paper (glass fiber filter type E from Gelman Co.) was then transferred into a scintillation vial with 10 ml of scintillation fluid (38) and the radioactivity was counted on a liquid scintillation counter (model 8260, Nuclear Chicago). The total incorporation of KCNO into phosphorylase a or b was calculated from the specific radioactivity of the chemical and the actual ^{14}C count in the TCA precipitated protein.

Amino acid analysis and hydrolysis of the cyanated enzyme protein was carried out as follows.

To samples of 1 ml containing 6-7 mg of protein were added 1 ml of 12 N HCl. The solutions were frozen in a dry ice-acetone mixture and the tubes were sealed under vacuum. Hydrolysis was then carried out in a 110°C oven for exactly 22 hours. The tubes were cooled before opening and dried in an evaporating mixer at 45°C. The amino acid analyses were carried out with the aid of an automatic recording Amino Analyzer (model 120B Beckman). In order to separate homocitrulline completely from valine,the 30-50°C system was employed (34).

Ultracentrifugal studies of sedimentation coefficients were determined with a Spinco model E analytical ultracentrifuge equipped with an RTIC unit for temperature regulation, employing a 12 mm single sector Kel F cell at rotor speed of 59,780 r.p.m. (250,000 x g). The temperature of the rotor during most runs was maintained at 20 ±

$2^{o}C$. Photographs of sedimenting boundaries were taken at recorded intervals during centrifugation. The buffer system was pH 6.8 with 1% KCl (or 1% K^{+} ion), 1% glycerophosphate, 0.06% Versene. The protein concentration was about 4-7 mg/ml. Movement of peaks was calculated from direct microcomparator measurements of the schlieren diagram. The percentage of components with different sedimentation coefficients was determined by estimation of areas of empirically resolved components. The sedimentation constant was calculated from the integrated equation:

$$S = \frac{\ln \frac{X_2}{X_1}}{\omega^2 \ (t_2 - t_1)} \qquad [2]$$

Where X_2 and X_1 are distances in cm from the center of rotation to the boundary at time t_2 and t_1 and ω is the angular velocity in radians per second (50). To obtain the standard sedimentation constant, $S^{o}_{20,w}$ is corrected as shown below.

$$S^{o}_{20,w} = S \times \frac{\eta_{b,t}}{\eta_{b,20}} \times \frac{\eta_{b,20}}{\eta_{w,20}} \times \frac{(1-\bar{V}_{20}\rho_{w20})}{(1-\bar{V}_{t}\rho_{t})} \qquad [3]$$

Where $\frac{\eta_{b,t}}{\eta_{b,20}}$ is the ratio of the viscosity of the buffer at $t^{o}C$ to its viscosity at $20^{o}C$; $(\eta_{b,20} = 1.01838)$

$\frac{\eta_{b,20}}{\eta_{w,20}}$ is the viscosity of the buffer relative to water at $20^{o}C$;

$\bar{V}_{20}$ and $\bar{V}_t$ are the partial specific volumes of the protein at 20°C and at t°, respectively; ($\bar{V}_{20}$ = 0.749)

$\rho_{w,20}$ and ρ_t are the densities of water at 20°C, and buffer at t°C, respectively. (ρ_{20} = 1.01011)

Pyridoxal-5-phosphate was liberated from phosphorylase by extraction with 0.3 N perchloric acid (PCA) with occasional agitation for 15-20 minutes at room temperature, followed by centrifugation. The PLP was stable under these conditions. The spectra of 0.3 N PCA extracts were determined under acid, neutral and alkaline conditions.

The preparative reduction of the Schiff base of PLP on phosphorylase with $NaBH_4$ was carried out as follows: a small beaker (10 ml) was set up in an ice bath and was filled to approximately one third with a solution of the enzyme (25 mg/ml) previously dialyzed against 10^{-3} M Versene, pH 6.8, overnight. A magnetic stirring bar was set in the beaker to control the mixing speed. Saturated neutral $(NH_4)_2SO_4$ solution was added to make up 33% saturation. The pH was then adjusted to 4.5 with ice cold acetic acid (0.2 M) and 0.25 ml of 10 mg/ml freshly prepared $NaBH_4$ was added at once. Under such conditions the bond to the PLP is converted almost entirely into a relatively stable secondary amine, resulting in instant decolorization of the bright yellow protein and leaving an insoluble product. The reaction was very fast and the reduced enzyme was centrifuged within one minute. The reduced enzyme was dissolved in 10^{-3} M Versene buffer, pH 6.8.

Disc electrophoresis chromatography - the apparatus

for this technique has been designed primarily for analytic quantities of human serum proteins or similar mixtures by Davis and Ornstein (35). The apparatus used was made in our laboratory according to the directions of Davis and Ornstein (35). A 0.02-0.03 ml sample of the enzyme preparation (10 mg/ml) was subjected to disc electrophoresis in a freshly prepared (5%) polyacrylamide gel column contained in a glass tube (5 mm x 7 cm) on the disc electrophoresis apparatus (35). The entire apparatus was refrigerated at 4°C and the voltage was kept at 115 volts between gel ends for about 1.5 hours. Bromphenol blue was added to the tris-glycine buffer, pH 8.2 (35) and the run was considered complete when the dye had passed through the gel column. After removal from the glass tubes, the gels were stained with 1% amido black 10B in 7% acetic acid for exactly 3 minutes and destained by washing with 7% acetic acid.

III. EXPERIMENTAL RESULTS

A. Kinetic Studies on the Inactivation

1. The course of the inactivation with time

It was found that buffers of 0.02 M glycerophosphate in 0.0015 M Versene or in 0.03 M cysteine were inadequate to maintain a constant pH when 0.1 M KCNO was added to phosphorylase. Typically, the pH increased from 6.8 to 8.5 during the reaction. In order to maintain the optimum pH for phosphorylase activity, the glycerophosphate concentration was increased to 0.1 M. Under this condition, the pH remained at 6.8 and the control enzymatic activity was very stable for 24 hours. To determine the inhibition of phosphorylase by KCNO, the phosphorylase and 1 M KCNO in 0.1 M glycerophosphate - 0.0015 M Versene buffer, pH 6.8, were preincubated in a 30°C water bath for about 10 minutes. The carbamylation reaction was started by the addition of KCNO to the enzyme solution to make a concentration of 0.1 M KCNO. The enzymatic activity of aliquots diluted one hundred times in 0.02 M glycerophosphate - 0.0015 M Versene buffer solution, pH 6.8, was measured at zero time and at various intervals. The inactivation of phosphorylase a by 0.1 M KCNO is demonstrated in Figure 2. The phosphorylase activity was measured in the direction of glycogen synthesis by determining the inorganic phosphate released by phosphorylase action in the presence and absence of adenylic acid, as noted previously in Methods. The control activities were determined at the same time on phosphorylase samples which

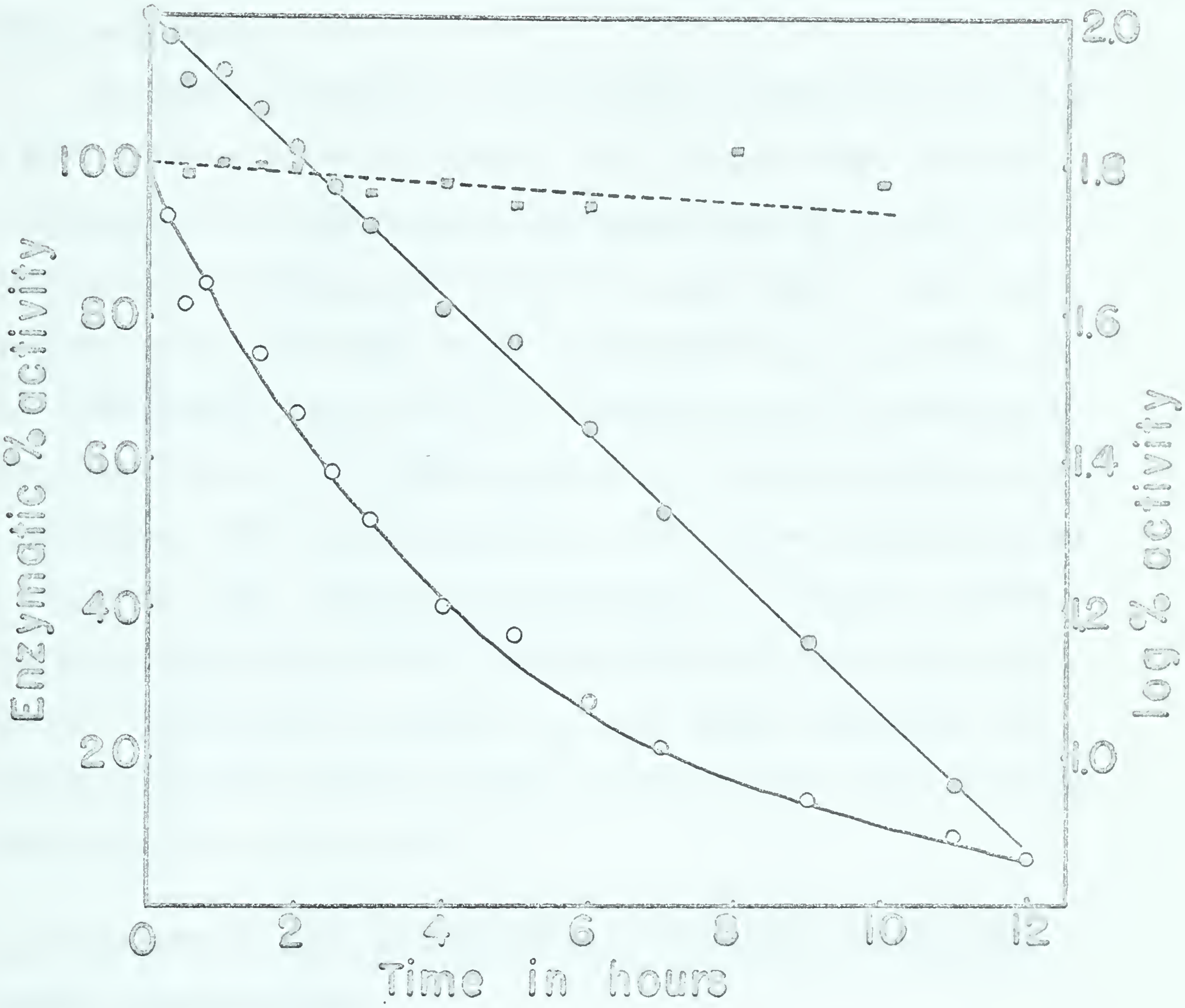


Figure 2. Inhibition of phosphorylase a activity by 0.1 M KCNO in 0.1 M Na-glycerophosphate - 0.0015 M Versene, pH 6.8. Enzyme concentration is 6.4 mg/ml.
○ enzyme activity % vs. zero time activity in 0.1 M KCNO
○ logarithm of enzyme activity % in 0.1 M KCNO
▪ control enzyme activity in 0.1 M KCl.

had been treated as above, with the exception that KCl was added instead of KCNO in order to maintain an equivalent ionic strength.

As shown in Figure 2, the enzymic inactivation by 0.1 M KCNO follows kinetics typical of a first order reaction. A comparison of rabbit muscle phosphorylase a, b and reduced phosphorylase a and b (20) has been made. The four enzymes have been shown to be inactivated by 0.1 M KCNO in the same manner except that the inactivation of phosphorylase b is faster than phosphorylase a, reduced phosphorylase a is faster than phosphorylase b, and reduced phosphorylase b is faster than reduced phosphorylase a. Figure 3 shows the rate constants for the inactivation of these four enzymes. Part of the increase in first order constants obtained with the reduced enzymes is due to the lower protein concentrations (see below).

2. Variation of the rate of inactivation with cyanate and enzyme concentration

The first order equation is $K't = 2.3 \log \frac{a}{a - x}$ and the reaction rate constant can be calculated by plotting the logarithm of the reciprocal of enzymic activity relative to the initial activity vs. time. The half time can be obtained from the equation $t \frac{1}{2} = \frac{0.69}{K'}$. The first order rate constants and half times were determined on individual experiments at varying concentrations of protein and KCNO. A deviation in rate constants was found, so the following experiment was tried: 1) a constant concentration of 0.1 M

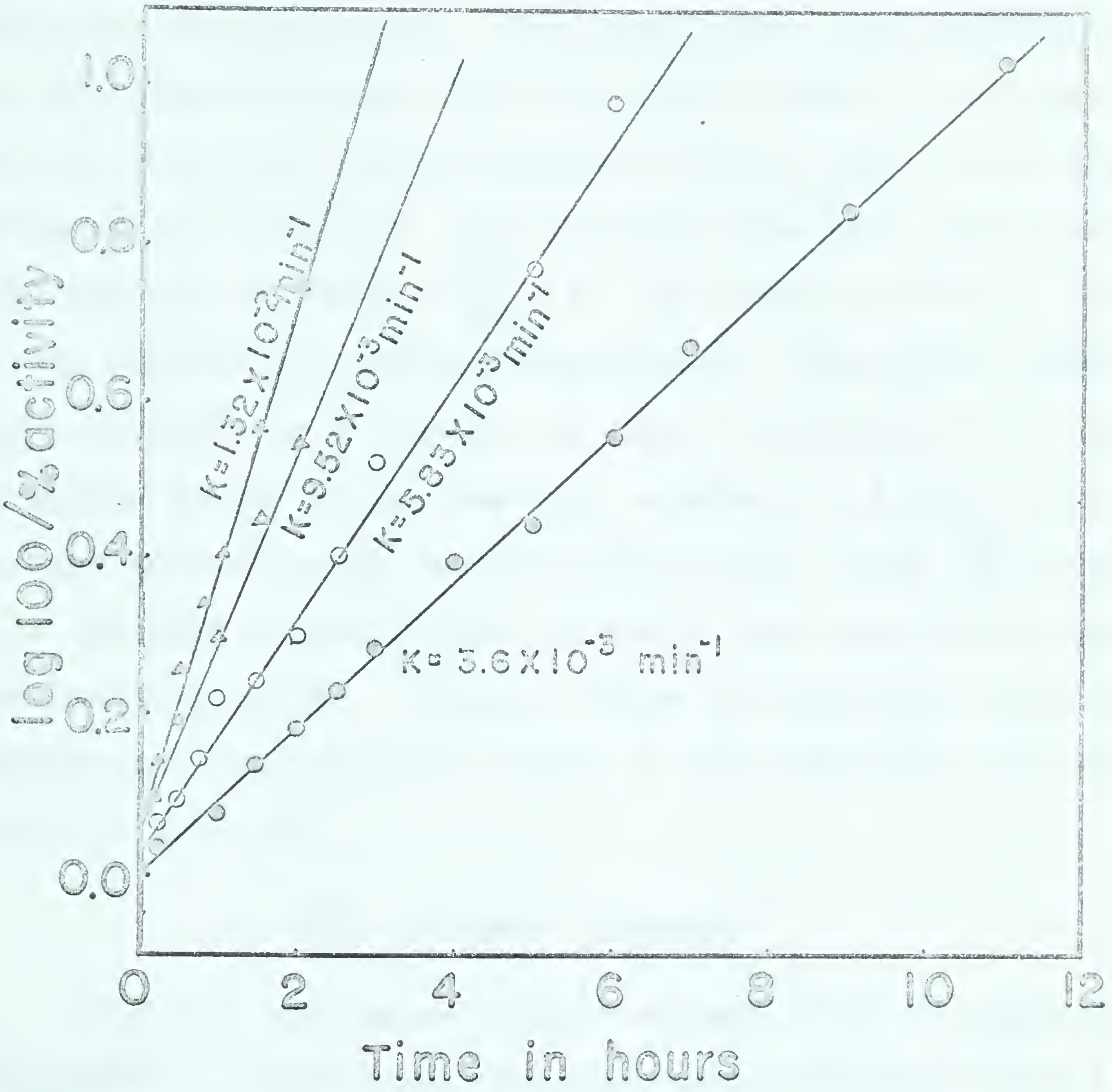


Figure 3. Comparison of phosphorylase *a*, *b* and reduced phosphorylase *a* and *b* with respect to their inactivation by 0.1 *M* KCNO in 0.1 *M* Na-glycerophosphate - 0.0015 *M* Versene buffer, pH 6.8.

- ⊙ phosphorylase *a* (6.4 mg/ml)
- ○ phosphorylase *b* (7.0 mg/ml)
- △ reduced phosphorylase *a* (1.9 mg/ml)
- ▲ reduced phosphorylase *b* (2.1 mg/ml)

KCNO was reacted with varying concentrations of phosphorylase a (0.5-8 mg/ml) and the inactivation for each enzyme concentration was determined. The first order rate constants were calculated as above and the results showed a decrease in the rate constant, and an increase in half time, as the enzyme concentration increased. The initial rates were calculated according to the formula $\frac{dx}{dt} = K'$ (mg protein per ml). When $\frac{dx}{dt}$ was plotted vs. enzyme concentration, there was a hyperbolic curvature relationship, as shown in Figure 4; 2) when the enzyme concentration was kept constant (4.8 mg/ml) and the KCNO concentration varied (0.03-0.15 M), then $\frac{dx}{dt}$ increased as the KCNO concentration increased, and there was a linear function established. Figure 5 shows the expected effect of changing the concentration of one of the components of a bimolecular reaction.

$$\text{i.e. } \frac{dx}{dt} = K(\text{KCNO})\ (\text{enzyme})$$

Here K is the second order constant which is equal to $\frac{K \text{ 1st order}}{[\text{KCNO}]}$. The second order constant was found to be 2.0, 3.2, 3.25, 3.25 and 3.30. L/M/min for .03, .05, .08, .1 and 0.15 M KCNO, respectively. This constancy of the second order rate constant at various concentrations of KCNO indicated that the rate of inactivation is directly proportional to the concentration of KCNO. It may also indicate an inverse proportionality of amount of activity to the amount of KCNO reacting.

The peculiar effect of protein concentration on the rate of inactivation may mean that the reaction is less than

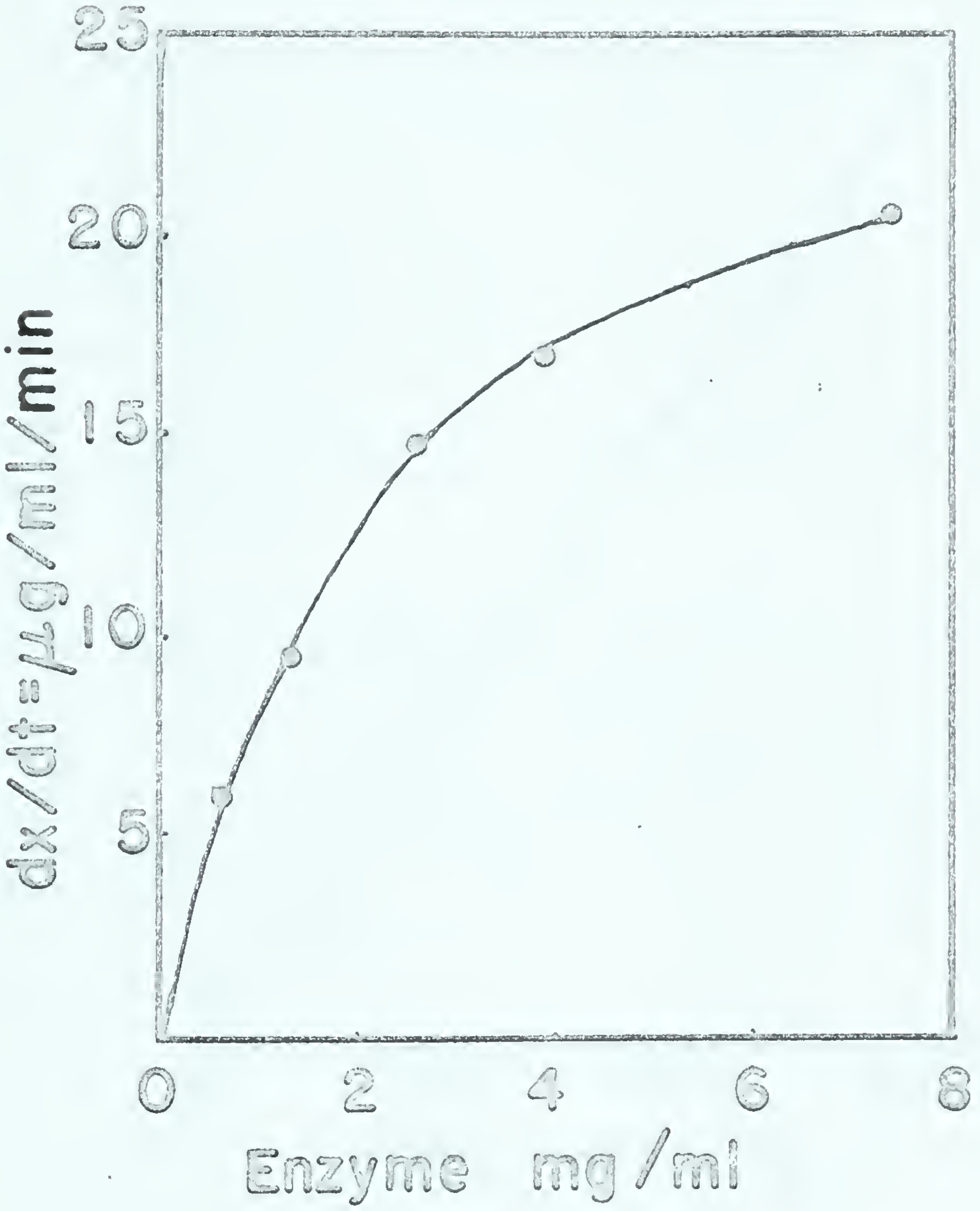


Figure 4. The effect of phosphorylase a concentration on the inactivation rate by 0.1 M KCNO in 0.1 M Na-glycerophosphate - 0.0015 M Versene buffer, pH 6.8. $\frac{dx}{dt}$ represents the inactivation rate.

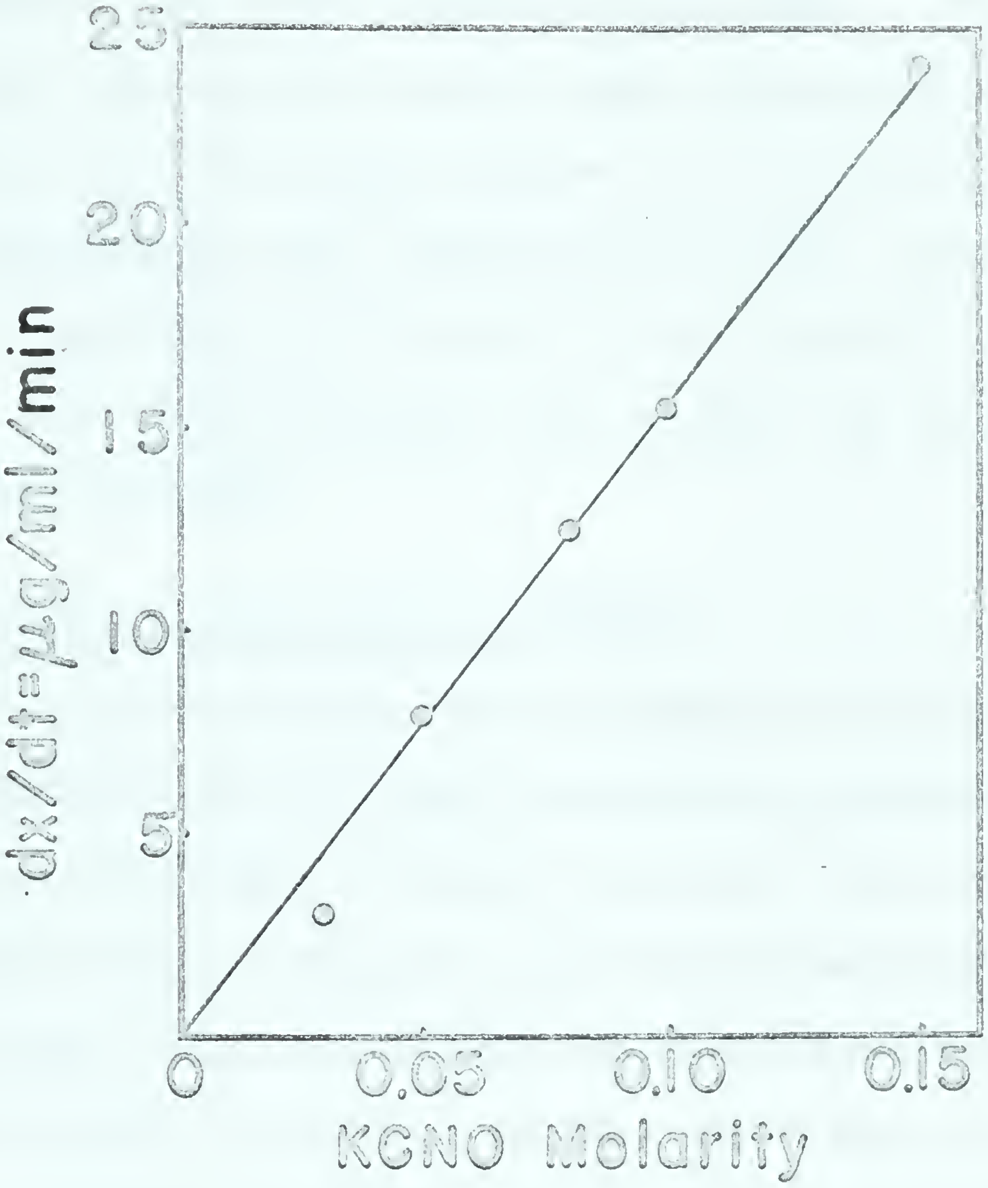


Figure 5. The effect of KCNO concentration on the inactivation rate of phosphorylase a (4.8 mg/ml) in 0.1 M Na-glycerophosphate - 0.0015 M Versene buffer, pH 6.8. $\frac{dx}{dt}$ represents the inactivation rate.

first order with respect to enzyme. This may be due to increasing protein concentration resulting in decreasing the accessibility of the sites reacting with KCNO. One might speculate that the reagent reacts with a dissociated form of the enzyme which is always present in a very small proportion in equilibrium with associated oligomer. As the concentration of protein is increased, the proportion in the dissociated form would decrease even though its absolute quantity would increase.

3. The rate of incorporation of $K^{14}CNO$

It was reported above that phosphorylase reacted with 0.1 M KCNO at pH 6.8 causes enzymatic inactivation and that the inactivation follows the first order reaction. Upon ultracentrifugal analysis, the inactivated phosphorylases dissociated into more slowly sedimenting molecules. Thus it was decided to examine whether there was any interrelation between the KCNO incorporation into phosphorylase, enzymatic inactivity and dissociation of phosphorylase. Stark *et al* (36) did a similar experiment. They carbamylated ribonuclease and found the specific activity of ribonuclease to be a function of the number of lysine residues altered by carbamylation with KCNO.

In this experiment, the 1.0 M KCNO, radioactive $K^{14}CNO$ and enzyme solution (in 0.1 M glycerophosphate, 0.0015 M Versene buffer, pH 6.8) were preincubated at 30°C. The carbamylation reaction was started by adding the enzyme solution to the KCNO. The final composition of the reaction

mixture was:

1)	6.5 mg/ml phosphorylase a	2)	7 mg/ml phosphorylase b
	0.1 M KCNO		0.1 M KCNO
	0.035 mc/ml $K^{14}CNO$		0.01 mc/ml $K^{14}CNO$

The incorporation of $K^{14}CNO$ into carbamylated phosphorylases was determined by the procedures outlined in Methods. As shown in Figure 6, the enzymatic inactivation and the incorporation of cyanate into phosphorylase increased with the time of reaction.

The first order rate constant can be obtained by plotting log 100/% activity vs. reaction time, as mentioned previously. From the data of Figure 7, the inactivation of phosphorylase a has a first order rate constant 3.6×10^{-3} min^{-1}, while that of phosphorylase b has a rate constant of 6.14×10^{-3} min^{-1} (Figure 8). The first order rate equation can be expressed as follows:

$$k't = 2.3 \log \frac{a}{a - X}$$

and the same first rate constants were also obtained when the value of a is taken as 52 in phosphorylase a and 23 for b, where a represents the lysine* residues which will effect the enzymatic activity and X is the amount of lysine residues which have reacted with KCNO. The results are shown in Figures 7 and 8.

The number of lysine residues per mole of protein were

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* The amino acid residues with which cyanate reacts is assumed here to be lysine, anticipating the evidence for this which will be presented in section D.

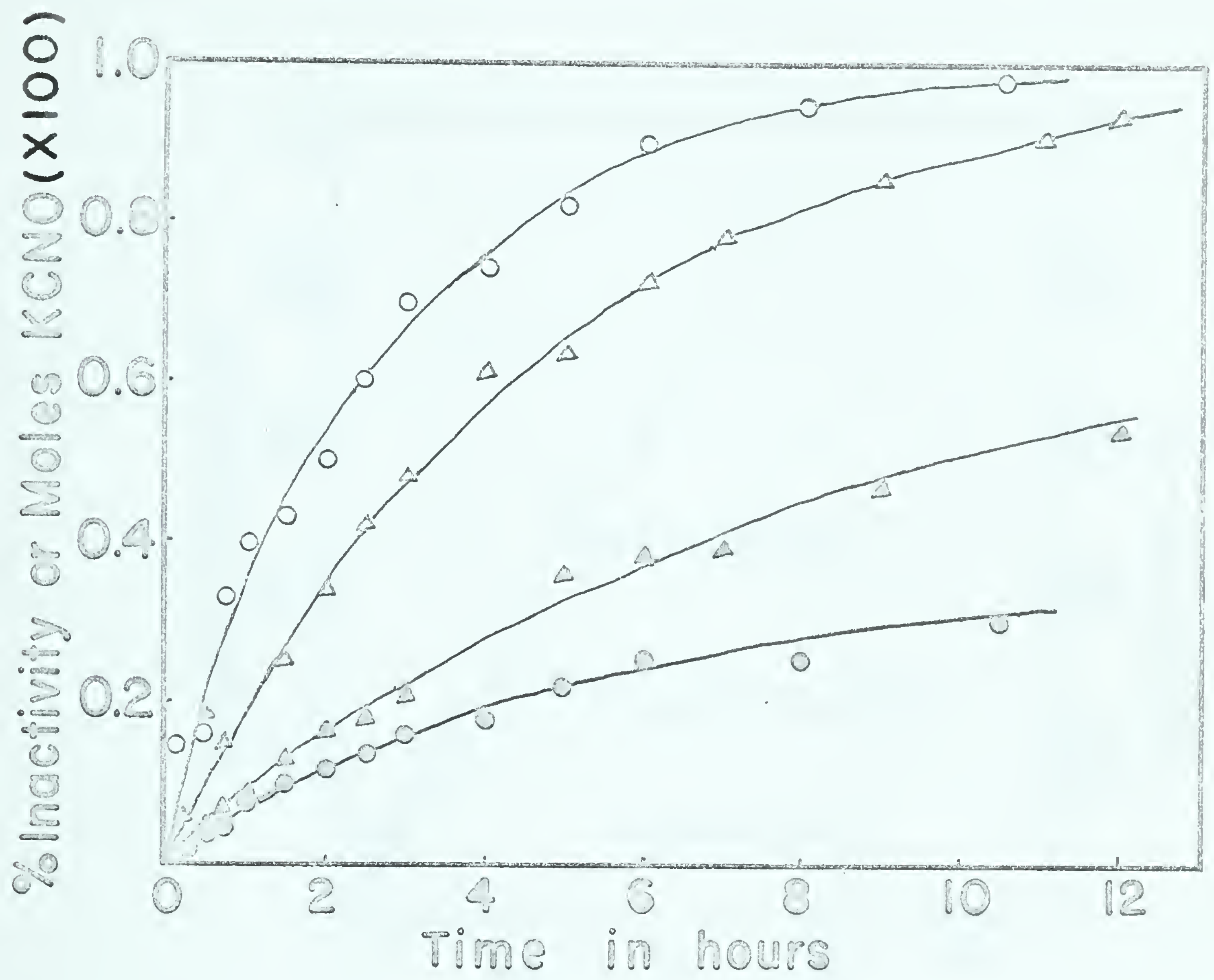


Figure 6. Comparison of phosphorylase a and b inactivation and K^{14}CNO incorporation into these enzymes in 0.1 M KCNO/ml concentration. Containing 0.05 mc K^{14}CNO/13 ml reactant. The buffer system is 0.1 M Na-glycerophosphate - 0.0015 M Versene, pH 6.8.

○ phosphorylase b (7.0 mg/ml) inactivity %
△ phosphorylase a (6.5 mg/ml) inactivity %
▲ moles of KCNO incorporated into each mole of phosphorylase b
◉ moles of KCNO incorporated into each mole of phosphorylase a in 0.1 M KCNO.

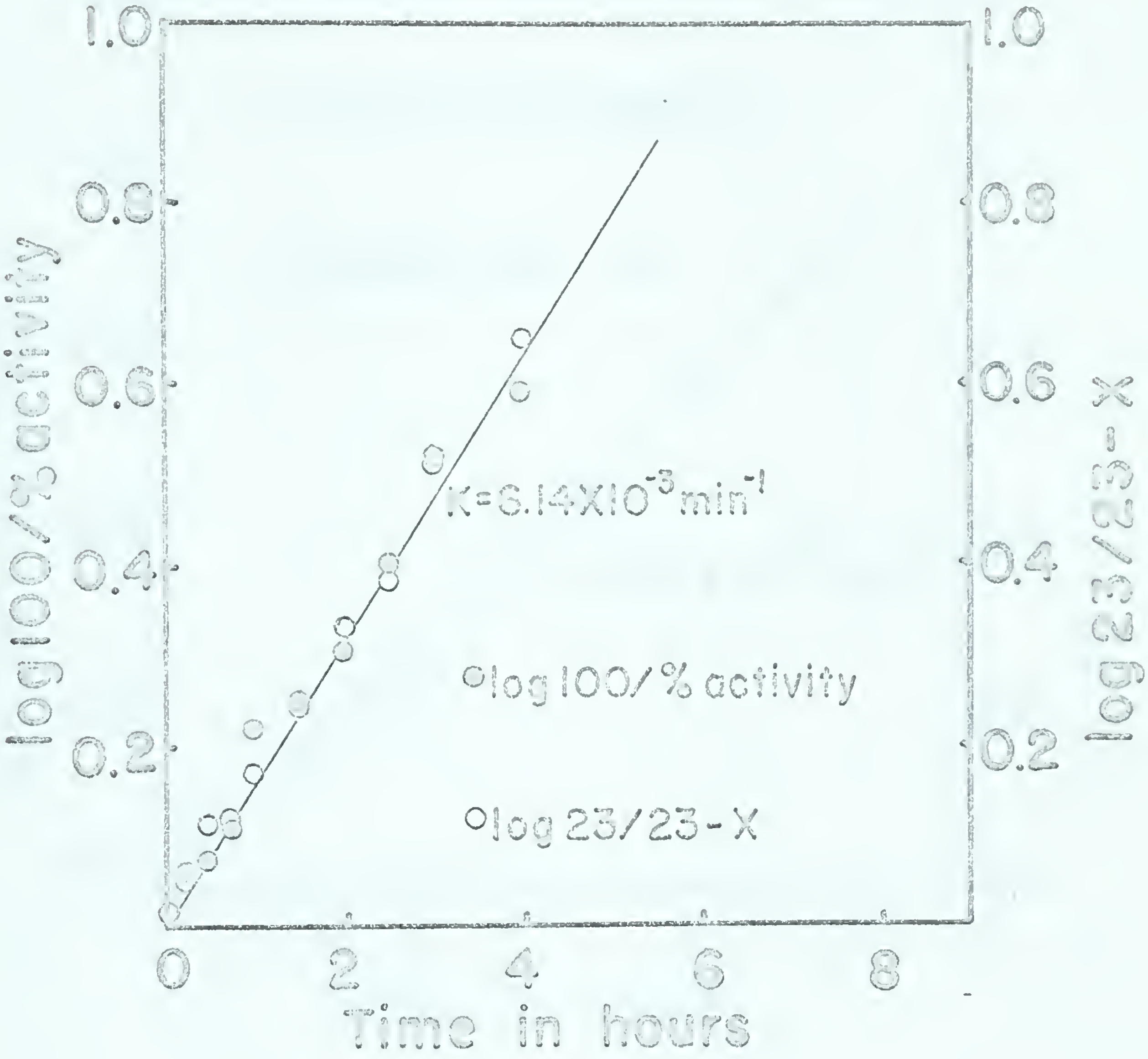


Figure 7. The first order rate constant calculated from both enzymatic inactivity and moles of KCNO incorporation into phosphorylase b. The experimental conditions are the same as in Figure 6.

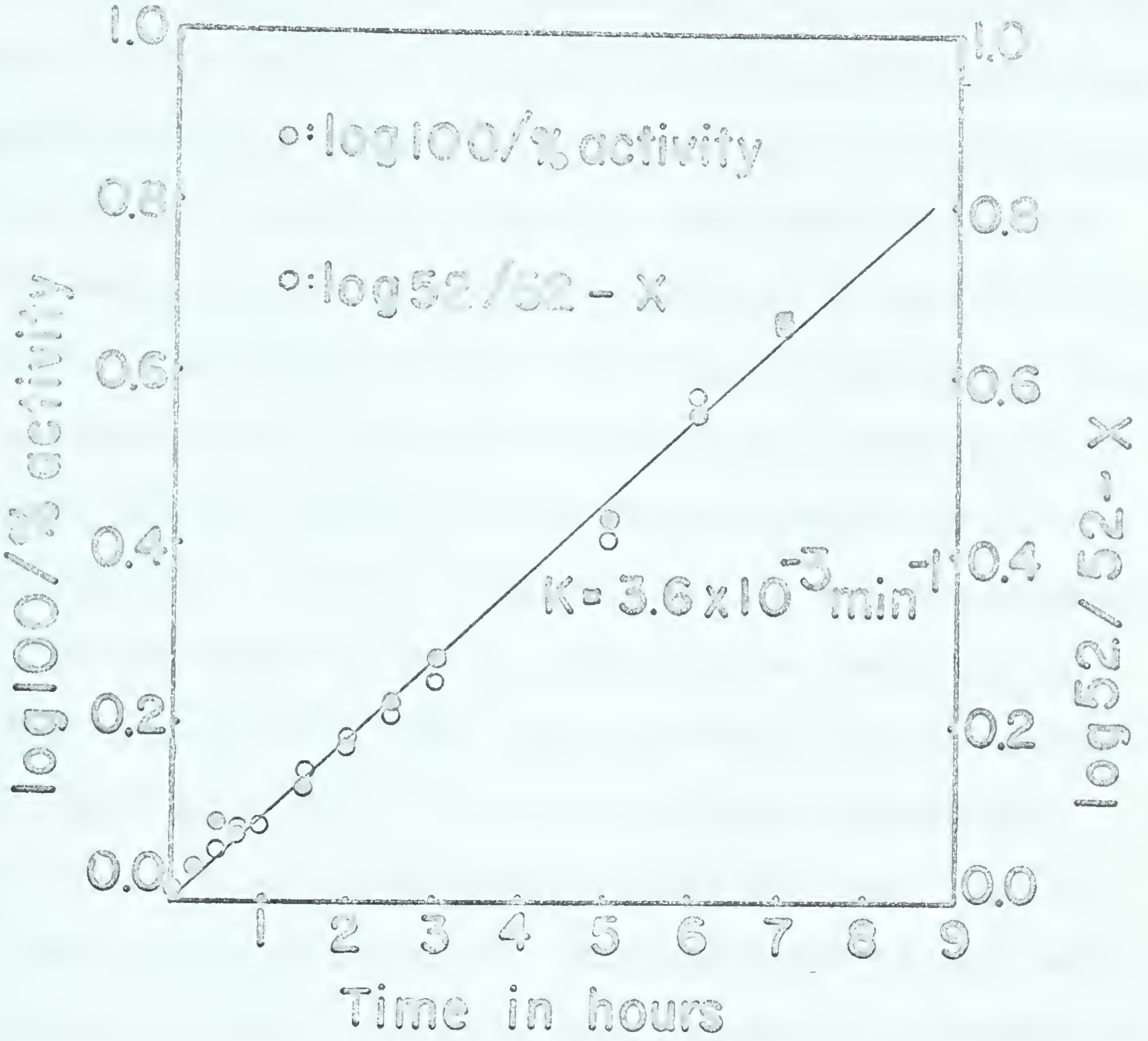


Figure 8. The first order rate constant calculated from both enzymatic inactivity and moles of KCNO incorporated into phosphorylase a. The experimental conditions are the same as in Figure 6.

assigned arbitrarily as being those which fit the data most closely. The value for phosphorylase a suggests that there are 13 lysine residues per monomer which are equivalent with respect to their effect on enzymic activity while the value for phosphorylase b suggests that there are 12 lysine residues per monomer in this category. The data for the two forms of phosphorylase are thus consistent in spite of the difference in molecular weight and rate of reaction. This does not necessarily imply that each lysine residue on an individual protein molecule contributes equally to the enzymatic activity. If the carbamylation is an "all-or-none" effect, as was shown to be the case for the reaction of sulfhydryl groups with PCMB, then one would expect a result such as that shown here. It has also been pointed out, however, that the opposite effect could be true, that is, if all the lysine residues are equivalent and if the carbamylation of each one causes a small change in conformation leading to changes in physical structure and activity, then one might see results such as are depicted here.

B. The Role of Sulfhydryl Groups

1. The effects of cysteine

Fraenkel-Conrat (49) and Stark (36) have shown that the sulfhydryl groups of cysteine and glutathione react rapidly with cyanate. Free sulfhydryl groups of phosphorylase have been well established by Madsen and Cori (16). They reported that inactivation of phosphorylase with PCMB is complete within 90 minutes and can be reversed by cysteine. Thus it was interesting to examine whether cyanate inhibition has the same behavior. The cyanate inhibition is different from PCMB inhibition in that it is much slower and it cannot be reversed with cysteine. If sufficient neutralized cysteine(in excess of the KCNO concentration) is added at any point during carbamylation, the enzymatic activity remains at a constant level (Figure 9). It would appear that the cysteine reacts rapidly with the free KCNO, thus preventing further carbamylation and enzymatic inactivation. It would appear that the carbamylation of phosphorylase was different from the PCMB reaction because the inactivation is not reversible with cysteine.

2. Evidence against the involvement of protein sulfhydryl groups in the inactivation of phosphorylase by cyanate

As mentioned above, the carbamylation inactivation could not be reversed with cysteine. On the other hand, it is remarkable that the PCMB titratable sulfhydryl groups of carbamylated phosphorylase *a* is equal to those of the uncarbamylated phosphorylase *a* when measured by Boyer's method

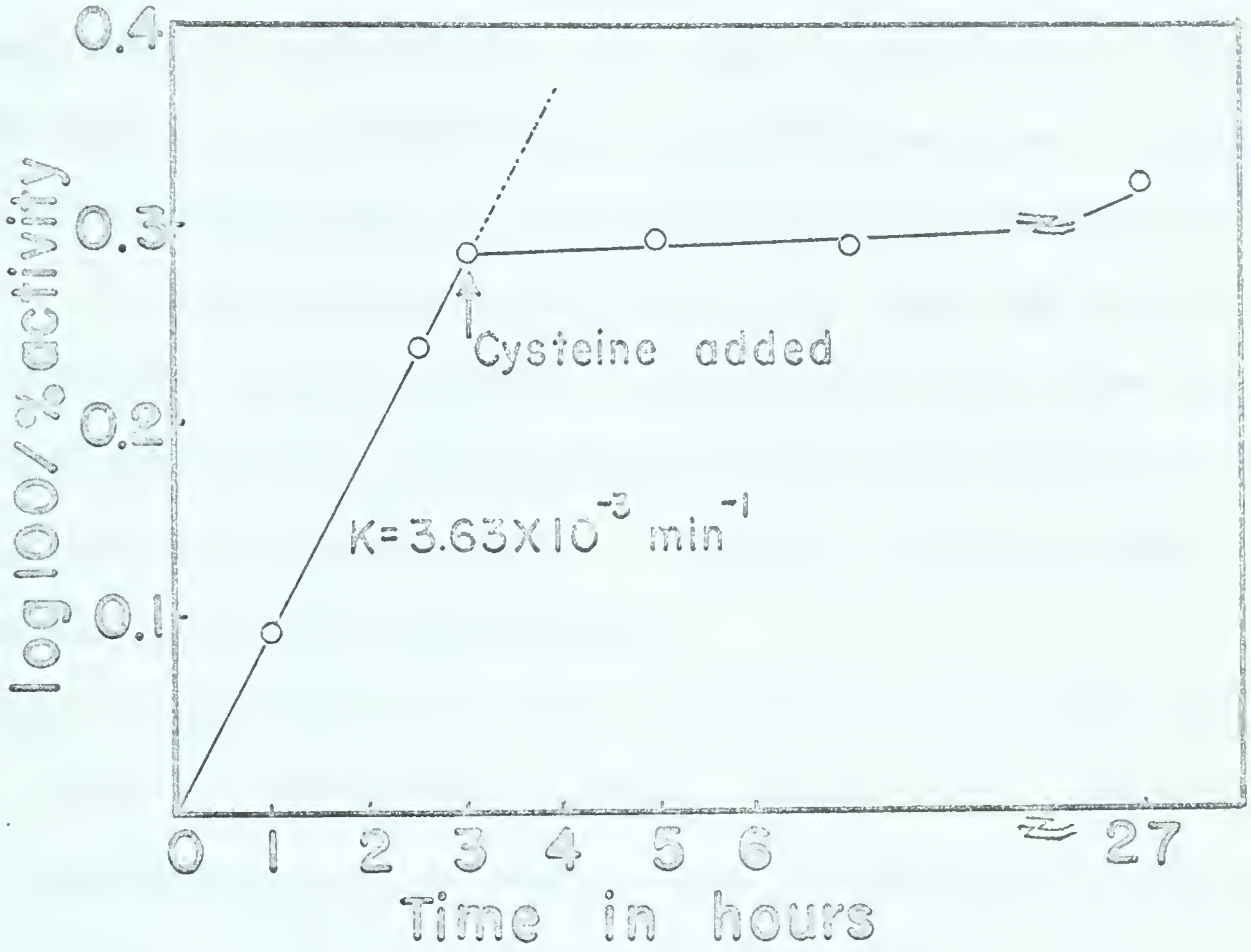


Figure 9. The effect of fresh neutralized cysteine to carbamylation inactivation of phosphorylase a, by 0.1 M KCNO in 0.1 M Na-glycerophosphate - 0.0015 M Versene buffer, pH 6.8. Phosphorylase a concentration is 7 mg/ml. At arrow point, fresh neutralized cysteine was added to a concentration of 0.1 M.

(37). 19.4 moles of PCMB titratable sulfhydryl groups were found in carbamylated phosphorylase a, which is close to the value of 18 moles per mole of phosphorylase a reported previously (16). Furthermore, the rate of reaction of PCMB with -SH groups of carbamylated phosphorylase a was similar to that previously found for the -SH groups of the native protein. It has also been found that the rate and extent of radioisotope carbon 14 KCNO incorporation into PCMB inhibited phosphorylase a and phosphorylase b was exactly the same as for the native proteins. Figures 10 and 11 show the data for these two experiments.

These experiments all tend to point to the same conclusion, that the immediate, primary cause of the inactivation of phosphorylase a by KCNO is not a reaction of the reagent with the sulfhydryl groups of the enzyme. This conclusion is reached in spite of the fact that KCNO reacts very rapidly with the free -SH groups of simple organic molecules (36,49). However, considerable evidence is available to indicate that the SH groups of phosphorylase are not freely available to react rapidly with reagents which would normally do so (16). Furthermore, Stark showed that the two -SH groups of β-lactoglobulin which do react with PCMB (41) do not react with KCNO except in the presence of 8 M urea (36).

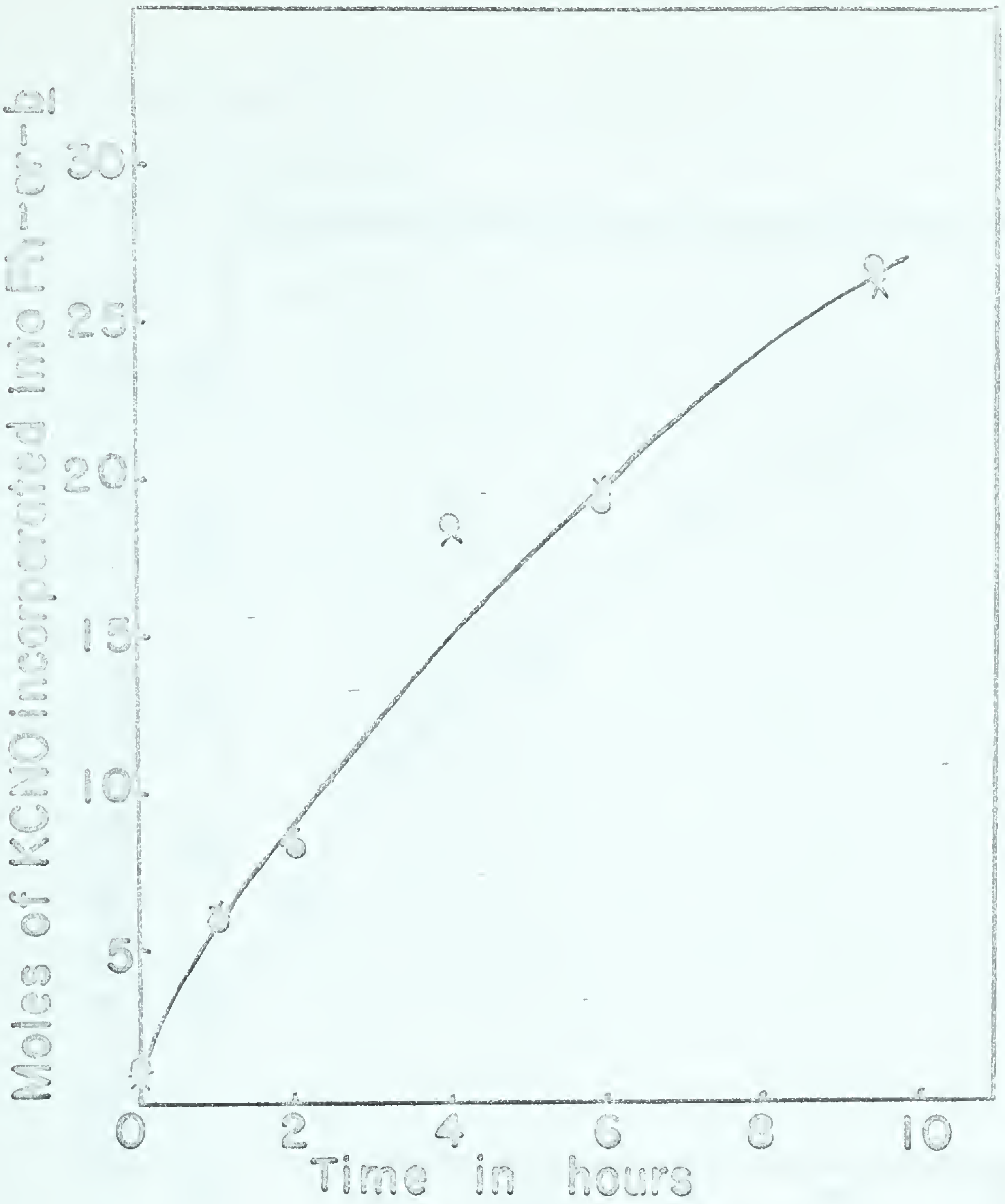


Figure 10. $K^{14}CNO$ incorporation into phosphorylase b and PCMB pretreated phosphorylase b (5.76 mg/ml) in 0.1 M glycerophosphate - 0.0015 M Versene buffer, pH 6.8. KCNO concentration was 0.1 M with 0.025 mc of $K^{14}CNO$. PCMB was 14 times the concentration of enzymes and was preincubated with enzyme at 30°C for 90 minutes before adding the KCNO. Aliquots of the reaction mixtures were transfered to neutralized cysteine buffer to stop the carbamylation reaction at the time shown. After dialyses against 0.02 M glycerophosphate-0.0015 M Versene buffer in a cold room overnight, the radioactivity was counted on a liquid scintillation counter on the 0.03 ml samples which were precipitated with 5% TCA and collected on millipore filter paper.

x—x represents the KCNO incorporated into normal phosphorylase b.

o—o represents the KCNO incorporated into PCMB pretreated phosphorylase b.

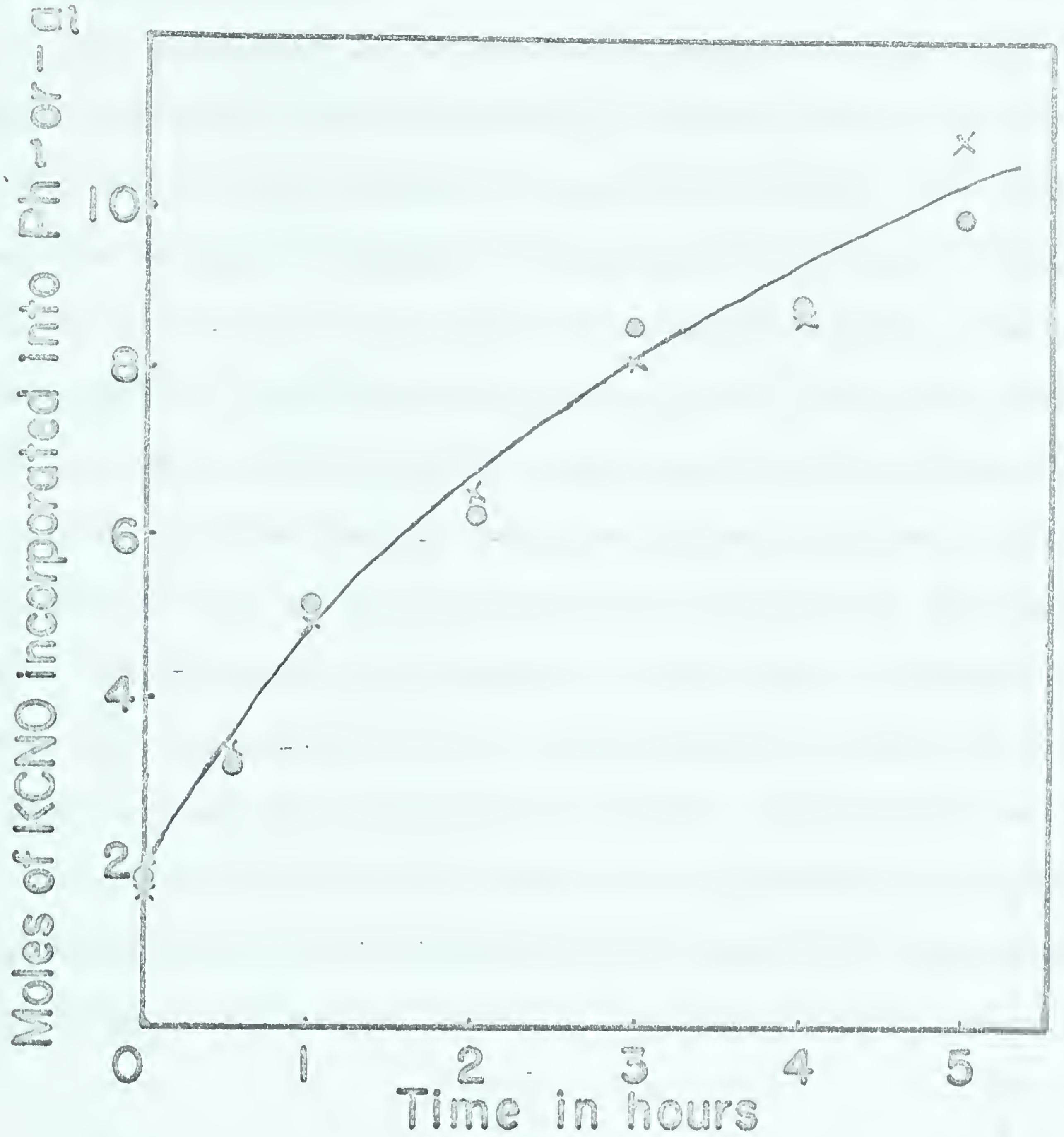


Figure 11. $K^{14}CNO$ incorporation into phosphorylase a and PCMB pretreated phosphorylase a (5.30 mg/ml) in 0.1 M glycerophosphate - 0.0015 M Versene buffer, pH 6.8. KCNO concentration was 0.1 M with 0.015 mc $K^{14}CNO$. PCMB was 22x concentration of phosphorylase a and incubated at 30°C for 90 minutes before adding KCNO and at this stage no enzymatic activity was shown. The radioactivity was counted on a liquid scintillation fluid counter on the trichloroacetic acid precipitate of aliquots.

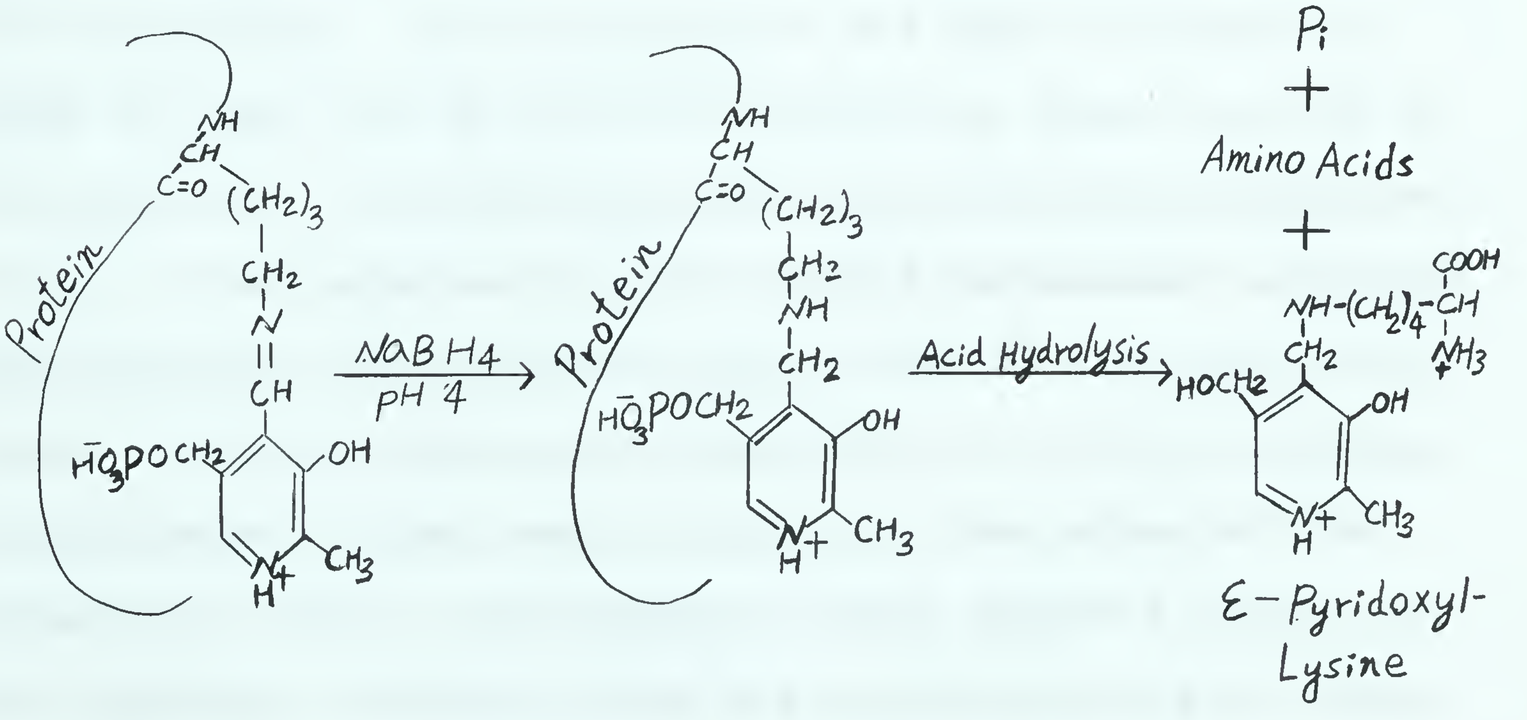
x—x represents the incorporation of KCNO into normal phosphorylase a.

●—● represents the incorporation of KCNO into PCMB pretreated phosphorylase a.

C. The Role of Pyridoxal-5-Phosphate

1. Spectral studies

It was noted by Krebs and Fischer in 1956 (12) that highly purified phosphorylase b solutions had a distinct yellow color when viewed in artificial light. In addition, when the enzyme in highly concentrated solution (7 mg/ml or more) was observed in the near ultraviolet region, the spectrum showed a well-defined peak at 333 mμ and the absorption was only about 1/20 the magnitude of the protein peak at 278 mμ in the freshly prepared phosphorylase b and a. The peak at 333 mμ is characteristic of PLP on the enzyme (54). Furthermore, the nature of the imine linkages between PLP and several of its apoenzymes has been clarified by Fischer and his associates (20,52). They have isolated ε-pyridoxyllysine from crystalline phosphorylase and from glutamate-aspartate-transaminase by reducing these enzymes with $NaBH_4$ followed by acid hydrolysis as shown below.


Protein
NH
CH
C=O
(CH2)3
CH2
N
CH
H̄O3POCH2
OH
N+
H
CH3
NaBH4
pH 4
Protein
NH
CH
C=O
(CH2)3
CH2
NH
CH2
H̄O3POCH2
OH
N+
H
CH3
Acid Hydrolysis
Pi
+
Amino Acids
+
COOH
NH-(CH2)4-CH
NH3+
CH2
HOCH2
OH
N+
H
CH3
ε-Pyridoxyl-Lysine

The aldimine linkage between PLP and apoenzyme is thus to the lysine residues of protein through its formyl group and this feature is common to each of the PLP proteins.

It was reported that phosphorylase a contains 4 moles of PLP, and that the b form contains 2 moles (18). This coenzyme is essential for phosphorylase activity and can be released in 2.5 M NaCl at pH 6.8 concurrently with lost enzymatic activity; and the activity can be restored by removing NaCl and readdition of PLP to this enzyme. Since the PLP is linked to apoenzyme through lysine residues of protein and lysine residues were easily attacked by KCNO, as mentioned previously, it seemed of interest to examine whether PLP binding lysine was reacting with KCNO and releasing the PLP, thus causing enzymatic inactivation.

This possibility was studied by spectrophotometric means. The advantage of this method was based on the different spectral behavior of PLP in free form and when bound to the protein. The free form of PLP has an absorption peak at about 390 mμ while the bound PLP shows the peak at 333 mμ (53). If KCNO did attack the PLP binding lysine residue, it was expected that PLP should be released and would then exhibit the absorption peak at 390 mμ, and the 333 mμ peak should decrease as the absorption of 390 mμ increased. Furthermore if there were a change in the nature of the binding of PLP to the enzyme one would expect a change in the spectrum. However, this was not found to be the case. In this experiment, a high concentration of phosphorylase b (9.23 mg) was incubated at 30°C with 0.1 M KCNO (see Methods).

The spectrum from 240 mμ and 500 mμ was measured at intervals of two hours and five and one half hours after adding the KCNO. The spectra showed two peaks at 278 mμ and 333 mμ for both carbamylated and control phosphorylase b, but no peak was detectable at around 390 mμ or at other wavelengths measured. The absorptions remained constant at two, four, and five and one half hour periods of carbamylation reaction, as shown in Figure 12.

At these carbamylation periods, the enzymatic activities were steadily decreased, following the first order reaction, and the activities at 2, 4 and 5 1/2 hours of reaction were 47%, 19% and 14% of the original enzymatic activity. These results indicated that the PLP was still bound to the protein during the carbamylation reaction period. The carbamylation phosphorylase b has an extinction value of 0.625 at 333 mμ for a one per cent solution of protein while the native enzyme has an extinction of 0.621. These values agree well with those reported by Kent (20, 28), which are in the range of 0.52 to 0.63.

2. Recovery of pyridoxal-5-phosphate after carbamylation

Another method of measuring the PLP content of phosphorylase is to extract the protein with 0.3 N perchloric acid. In this method one always observes a transient bright yellow color and the acid extract has adsorption maxima at 295 mμ and 333 mμ. A permanent yellow color also showed in 0.1 N NaOH solution in which the peak shifted to 388 mμ (28). The splitting sequence of PLP from phosphorylase with

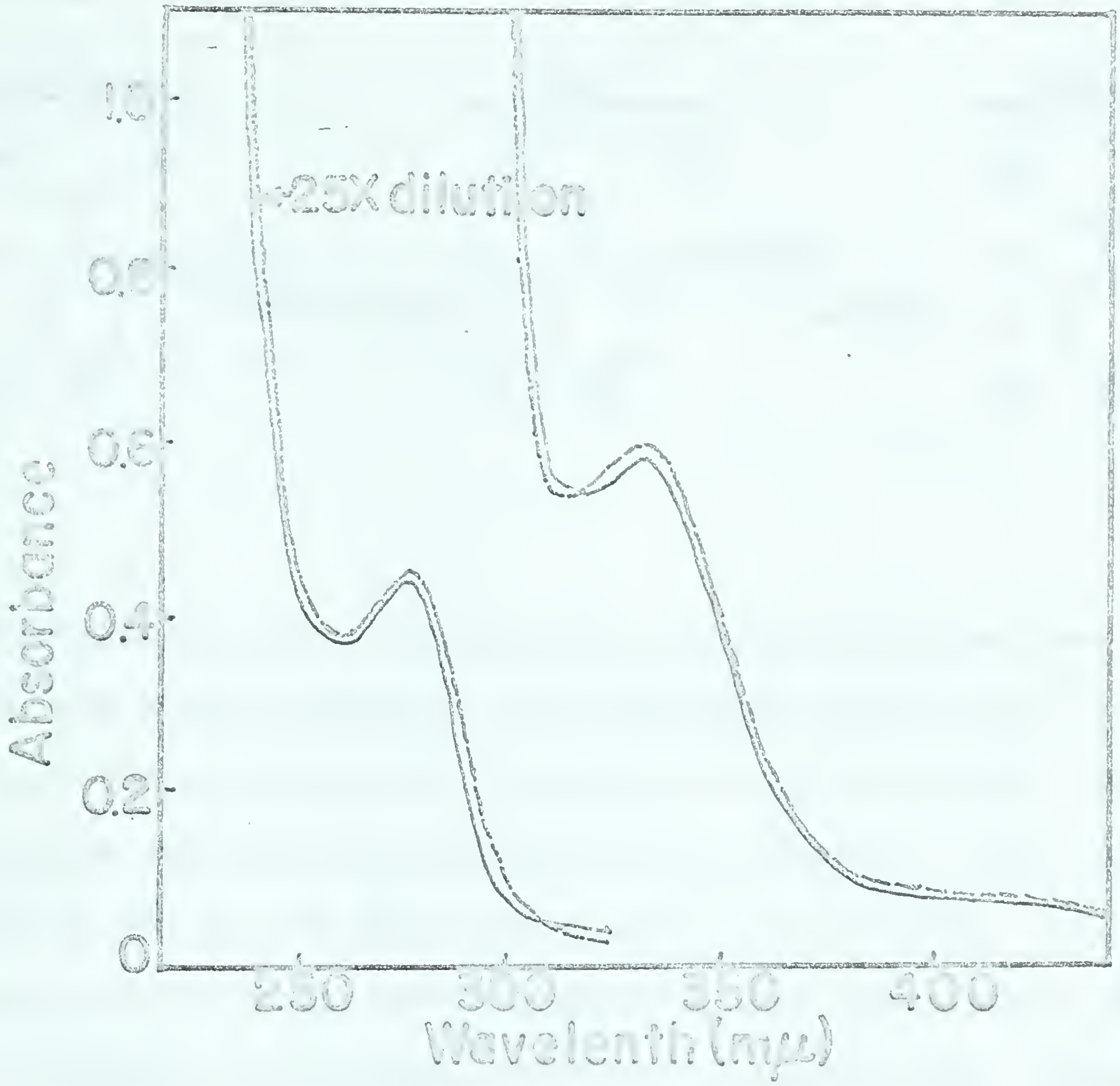
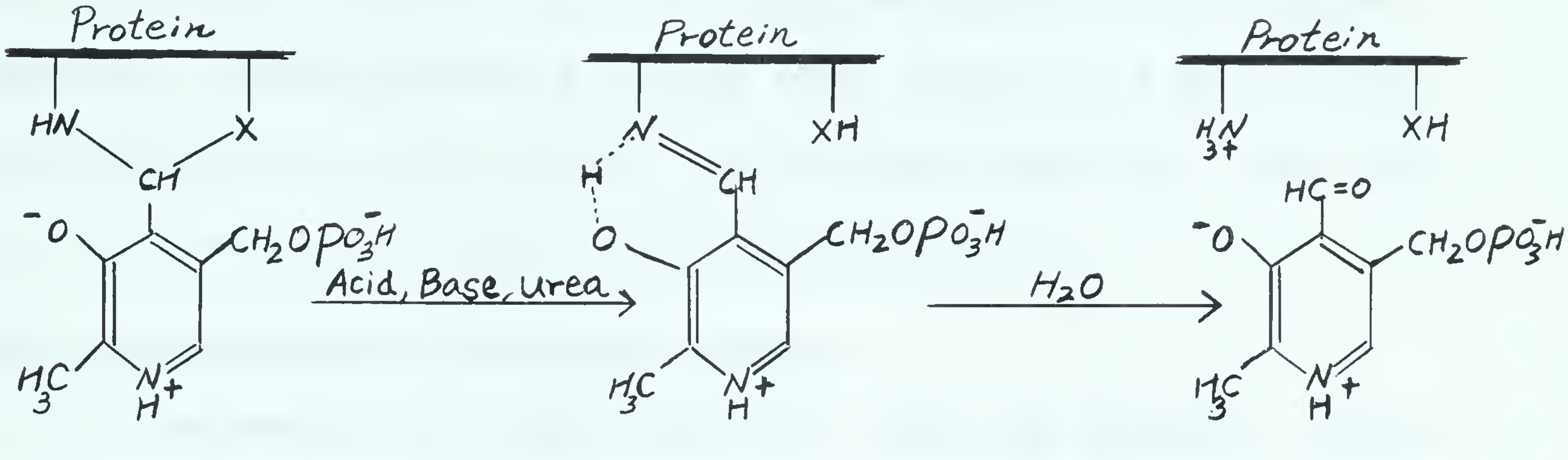


Figure 12. Spectra of carbamylated and control phosphorylase b after incubation with 0.1 M KCNO and 0.1 M KCl for five and one half hours at 30°C. Solid line represents phosphorylase b (9.23 mg/ml) with 0.1 M KCNO. Broken line represents control phosphorylase b (9.65 mg/ml) with 0.1 M KCl. $E^{1\%}_{333\ m}$ for control phosphorylase b = 0.62 and carbamylated phosphorylase b = 0.625.

acid or base, as suggested by Fischer and Kent, is shown as follows:



Carbamylated phosphorylase b and control phosphorylase b from the above experiment were dialyzed against 10^{-3} M Versene buffer, pH 6.8, for 24 hours in the cold room. The proteins were then extracted with 0.3 M PCA and the absorption at 295 mμ and 388 mμ measured in 0.1 N NaOH. The recovery of PLP from control phosphorylase b was about 98% as compared with Fischer and Kent's results (28). Carbamylated phosphorylase b also showed peaks at these two wavelengths, except that lower absorptions were obtained with recovery rates of 55-67%. Evidently some of the PLP in carbamylated phosphorylase was released during the 24-hour dialysis period. As shown by ultracentrifugal studies, the dimer from normal phosphorylase b was dissociated into monomer form during the latter stage of the carbamylation reaction. Graves and Wang also found that the pyridoxal phosphate is released from phosphorylase in 2.5 M NaCl following dialysis against buffer solution at pH 6.8. In this case, the tetrameric form of phosphorylase a was dissociated

into a dimeric form (25). It seems that the PLP in the dissociated phosphorylase was much more unstable than in the natural form. Thus it is not surprising that the cyanate modified phosphorylase b became more labile and more prone to releasing its PLP during the 24-hour dialysis, resulting in low recovery of PLP by acid extraction.

3. Inactivation of reduced enzyme

The method of Krebs and Kent (28) was used for reducing phosphorylase preparations, as mentioned in Methods. The reduction by $NaBH_4$ occurred only at the pyridoxal-5-phosphate which was reduced to pyridoxamine phosphate.

The spectrum of this reduced phosphorylase b was exactly the same as that reported by Krebs and Kent (28). However, the enzymatic activity was uniformly decreased by about 40-50% upon reduction. This effect did not seem to be related to the concentration of $NaBH_4$ in the assay system (ranging down to 0.005 mg/ml $NaBH_4$). The somewhat lower activity of reduced phosphorylase b may be due to its inherent instability. Krebs and Kent also found a 20% decrease in activity in reduced phosphorylase b. The reduced phosphorylase a and b were also studied in carbamylation reactions. The $NaBH_4$ treated enzymes were dialyzed against 10^{-3} M Versene, pH 6.8, in a cold room (4°C) overnight and used as enzyme sources. The carbamylation reaction was performed as described for previous experiments. It was found that the reduced enzymes were also inhibited by 0.1 M KCNO in 0.1 M Na-glycerophosphate - 0.0015 M Versene buffer, pH 6.8, and the inactivation rate also followed a first order reaction. The results are shown in Figure 3.

D. Amino Acid Analyses of Carbamylated Phosphorylases

The determination of the amino acid composition is an integral part of the study of the structure of any purified enzyme. The amino acid composition of rabbit skeletal muscle phosphorylase a was determined by Velick and Wicks (33) in 1951. These workers used isotope dilution, microbiological analysis and colorimetric methods. In 1962 Appleman (39) used the chromatographic method of Moore et al (40), with an automatic recording apparatus and spectrophotometric methods to determine rabbit skeletal muscle and human muscle phosphorylase b amino acids, which are shown in Table I.

The results of Velick and Wicks and those of Appleman were quite similar, with the exception of cysteine residues. Velick and Wicks found eighteen cysteine residues in phosphorylase a, which was in agreement with the P-chloromercuribenzoate titration of phosphorylase a (16). Appleman found 22 cysteine residues per mole of phosphorylase b, which would correspond to 44 cysteine residues per mole of phosphorylase a. Kudo and Shukuya (42) also reported 18 cysteine residues in phosphorylase a as measured by PCMB titration. It would appear that approximately 50% of the sulfhydryl groups in phosphorylase are not available for titration by PCMB, or else that there are unsuspected disulfide bonds.

In 1960 Stark et al (36) found that when ribonuclease was maintained in 8 M urea at 40°C and chromatographed on IRC-50, a large unexpected peak was revealed on the effluent curve. Upon amino acid analysis of acid hydrolysates of this sample, the lysine content was found to be less than that of

TABLE I

The amino acid composition of rabbit and human phosphorylases

Amino acid	Residues/mole of phosphorylase *b* (A)		Residues/mole of phosphorylase *a* x 0.5		Residues/mole of carbamylated phosphorylase *a* x 0.5	
	Rabbit	Human	Rabbit		Rabbit	
Lysine	126	123	109	126**	61	72**
Histidine	58	61	49	57	49	58
Ammonia	186	179	187	187	187	187
Arginine	164	166	143	166	141	166
Aspartic acid	235	246	220	254	208	245
Threonine	82	83	77	89	76	90
Serine	61	64	59	68	58	68
Glutamic acid	232	230	224	260	220	259
Proline	87	86	70	81	70	83
Glycine	116	120	101	117	100	118
Alanine	153	161	132	153	130	153
Half-cysteine	22	21	-	-	-	-
Valine	149	147	106	123	116	137
Methionine	52	56	47	54	46	54
Isoleucine	120	121	90	104	89	105
Leucine	196	196	176	204	173	204
Tyrosine	88	85	82	95	79	93
Phenylalanine	96	103	86	100	82	97
Tryptophan	30	29	-	-	-	-
Homocitrulline	0	0	0*	-	43*	51

(A) From data of Appleman *et al* (39).

* Homocitrulline (corrected for the 24% decomposed during 22 hours hydrolysis at 110°C).

** All values corrected on basis of adjusting data for alanine to that obtained by Appleman *et al*.

ribonuclease a and in addition, with a temperature adjustment from 30° to 50° at 280 ml of eluate, a new peak appeared which followed the valine peak. The amount of this new amino acid increased and the amount of lysine decreased as the time of exposure of ribonuclease to urea was lengthened. They concluded that the loss of lysine had come about as a result of carbamylation of the ε-NH_2 groups by the cyanate in urea solution. Warner (43) concluded that cyanate was the sole intermediate in the hydrolysis of urea to ammonium carbonate. Dirnhuber and Schütz (55) estimated that an 8 M urea solution would be 0.02 M in respect to cyanate. Stark et al (36) also showed the carbamylation of lysine in ribonuclease with KCNO and confirmed that this new compound was homocitrulline by a comparison of the chromatographic behavior of carbamylated ribonuclease and standard homocitrulline. Homocitrulline emerged at exactly the same position as the new peak from carbamylated ribonuclease.

The samples for amino acid analysis of carbamylated phosphorylases a and b (see Methods) were hydrolyzed with 6 N HCl for 22 hours at 110°C in evacuated sealed tubes and carried out with the aid of the automatic recording amino acid analyzer (Beckman model 120B) described by Moore and Stein (34). The 30°-50° system was employed and the eluent and temperature changes were made at 280 ml (36). From the experiment, results shown in Figure 13 indicate that the new peak appeared at the same position as the homocitrulline peak from carbamylated ribonuclease (36). The uncarbamylated phosphorylases a and b have no detectable peak at this position,

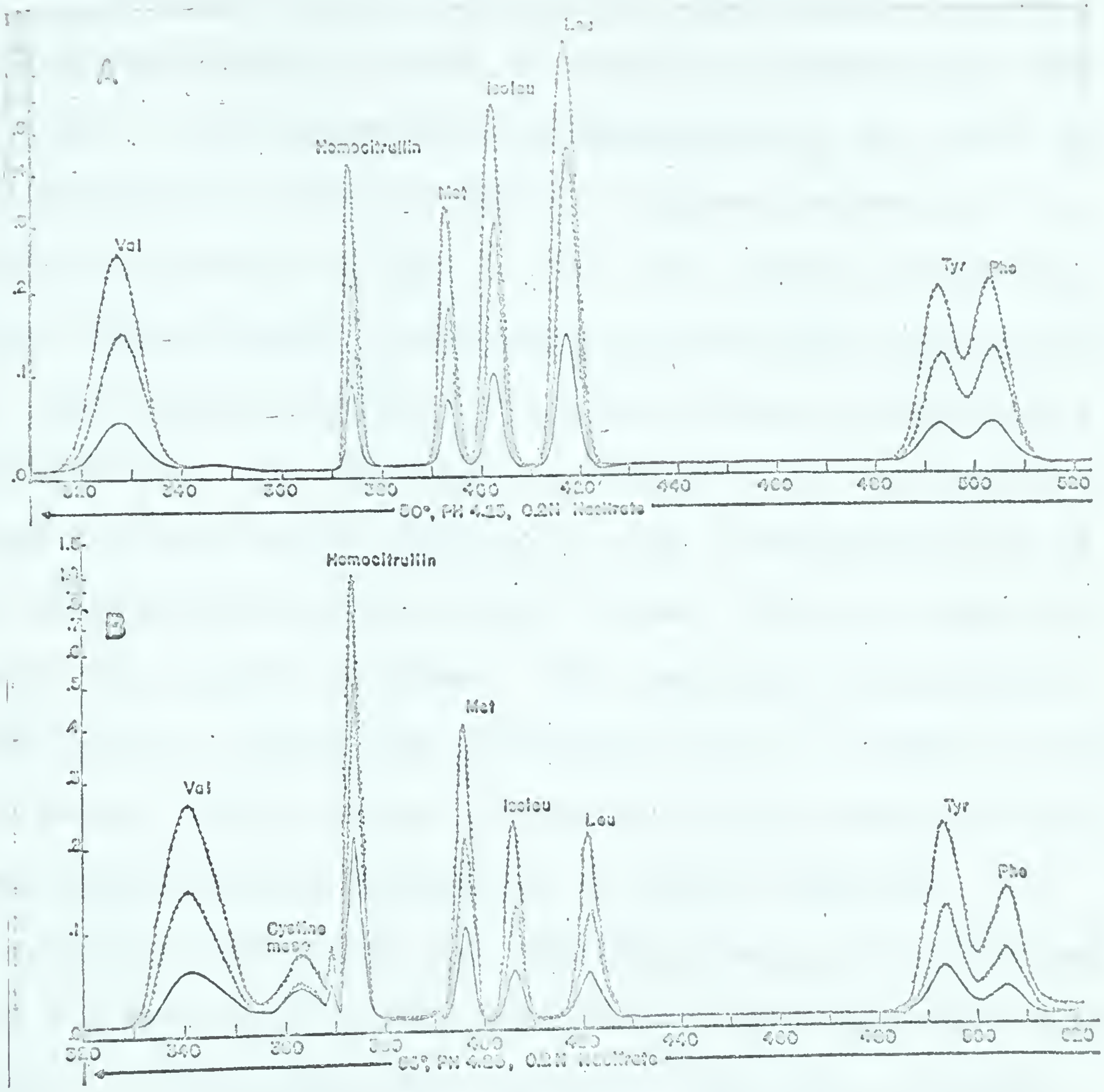


Figure 13. The comparison of an amino acid chromatograph pattern of the carbamylated phosphorylase a and automatic recording equipment were used, with a temperature change from 30° to 50° at 300 ml.

A. Phosphorylase a carbamylated with 0.1 M KCNO in presence of 8 M urea for 24 hours at room temperature.
B. Carbamylated ribonuclease from Stark, Stein and Moore (36).

thus it can be concluded that this new peak from carbamylated phosphorylase was homocitrulline. As illustrated in Table I, the total amino acids recovered from several samples under these conditions were 90 ± 3% of that reported by Appleman (39) when expressed in terms of number of residues per mole of protein. The carbamylated phosphorylase a for which the amino analysis is shown in Table I had been exposed to 0.1 M KCNO for 23 hours at pH 6.8, 30°. The amount of homocitrulline from different carbamylation conditions were as follows: the normal phosphorylase a and b have no detectable homocitrulline. Phosphorylase a incubated in 0.1 M KCNO for two and one half hours, leading to 42% inactivation has 20 moles of homocitrulline residues formed. This was equal to 10.6% of the lysine residues. The completely inactivated phosphorylase a, which was incubated with 0.1 M KCNO at 30°C for 23 hours, has 86 moles of homocitrulline residues which corresponded to 41.5% conversion of lysine residues. In more vigorous conditions, the phosphorylase a was incubated with 0.1 M KCNO in 8 M urea solution at room temperature for 24 hours. It was found that 46.2% of the lysine residues were converted into homocitrulline. In the case of phosphorylase b, the enzyme inactivated to 60% by 0.1 M KCNO has 9.1% of its lysine residues converted to homocitrulline. 27.5% of the lysine residues were converted to homocitrulline in completely carbamylated phosphorylase b and 40% conversion was found in samples of phosphorylase b incubated with 0.1 M KCNO-8 M urea for 24 hours at room temperature. From these results it seems that less than 50% of the lysine residues

of phosphorylases could be carbamylated even in the presence of 8 M urea and the amount of homocitrulline increased as the enzymatic inactivity increased. In another experiment, amino acid analyses for both phosphorylases a and b were done on a series of samples at various carbamylation times and it was found that the enzymatic activity decreased as exposing to 0.1 M KCNO was lengthened. In spite of a lower recovery of all amino acids, (about 68% recovery) the results showed that the amount of lysine residues decreased concomitantly with increasing the amount of homocitrulline residues.

E. The Effects of Substrates and AMP

1. The binding of AMP by carbamylated phosphorylase

It was felt that the carbamylation of phosphorylase resulting in enzymatic inactivation could be due to blocking of the active sites or binding sites. If this were so, then the substrate may protect against the carbamylation reaction, and carbamylated phosphorylase may lose the ability to bind the substrate. The binding of AMP to phosphorylase has been studied by both spectrophotometric and ultracentrifugal means (17). By the spectrophotometric method it was found that the carbamylated phosphorylase _a_ has lost most of the AMP binding characteristics of the native protein (Figure 14).

The rational of this approach depends on the decrease in the absorbance of AMP when it is bound to phosphorylase, accompanied by a slight shift in the spectrum. This is shown by the control experiment depicted in Figure 14. The carbamylated phosphorylase _a_ causes only a very slight shift in the spectrum of AMP. This may mean either that the dissociation constant for AMP and protein has been greatly increased, or that the nature of the binding has been changed markedly.

2. Effects on the rate of inactivation by cyanate

Possible protection against inactivation was tested by preincubating phosphorylase _a_ with the following at 30°C:

1) 1% glycogen
2) 0.016 _M_ glucose-1-phosphate
3) 0.001 _M_ AMP
4) 0.016 _M_ glucose-1-phosphate and 0.001 _M_ AMP

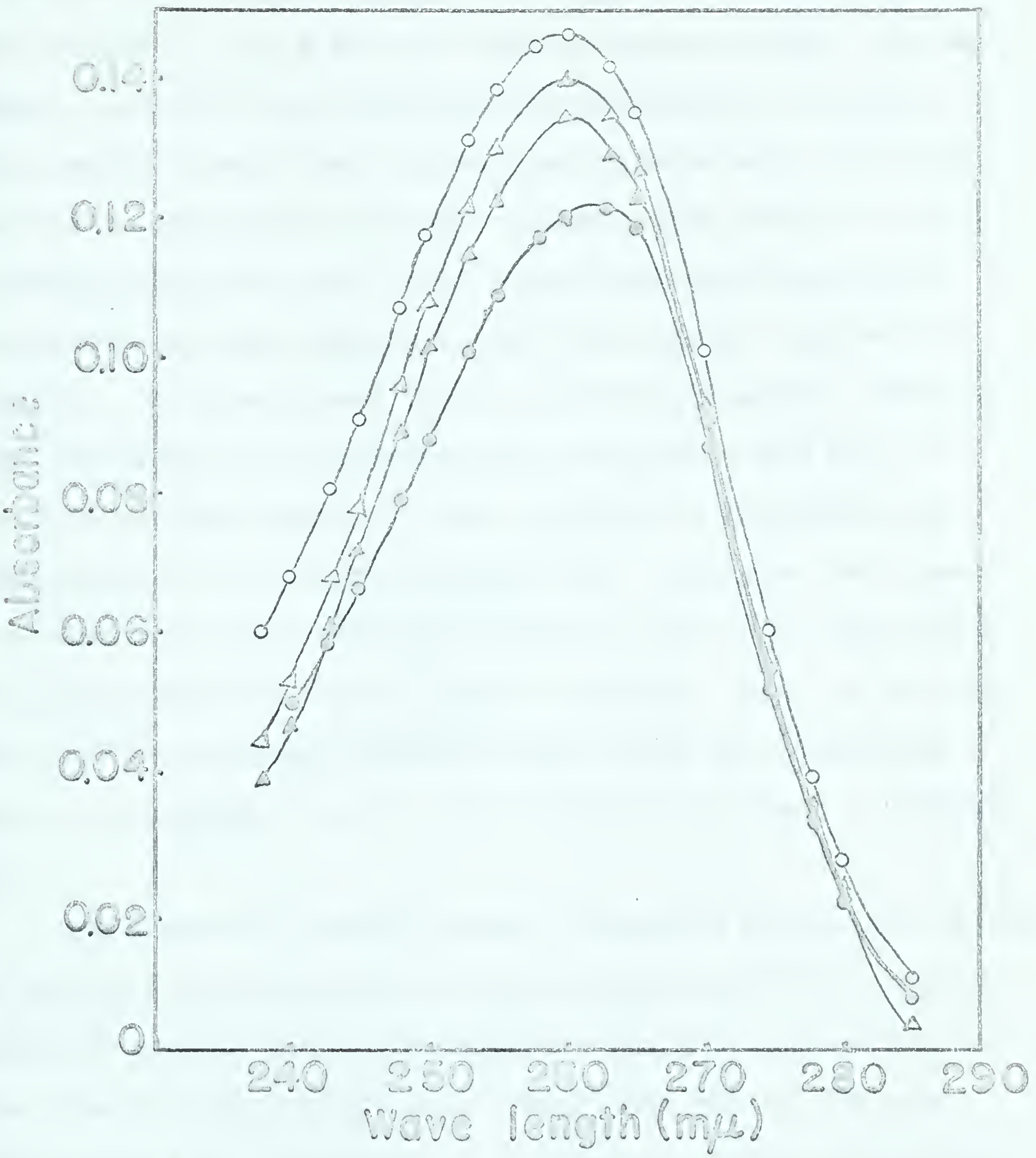


Figure 14. Comparison of phosphorylase a and 90% carbamylated phosphorylase a for the ability to bind AMP in 0.02 M Na-glycerophosphate - 0.0015 M Versene buffer, pH 6.8.

○ spectrum of 9.15×10^{-6} M AMP
● spectrum of 9.15×10^{-6} M AMP plus 1.178×10^{-6} M phosphorylase a
△ spectrum of 8.75×10^{-6} M AMP
▲ spectrum of 8.75×10^{-6} M AMP plus 2.43×10^{-6} M carbamylated phosphorylase a

After preincubation with these substrates, 1 M KCNO was added to a concentration of 0.1 M KCNO. In control enzyme solution, 0.1 M KCl was used to replace KCNO. The enzymatic activity was determined as mentioned in Methods. The results showed that enzyme preincubated with 1% glycogen had no protection against carbamylation, while 0.016 M glucose-1-phosphate and 0.001 M AMP provided significant protection against inhibition and had similar protective ability. It also showed that even more protection occurred when the enzyme was preincubated with both 0.016 M G-1-P and 0.001 M AMP together. The increase in protection by combining G-1-P and AMP together might indicate that these two substrates have separate binding sites and these sites have some relationship to lysine residues. This is not the case for the glycogen binding site, since this substrate gave no protection at all. The results are shown in Figure 15.

It should be noted, however, that the protection afforded by G-1-P and AMP against inactivation by KCNO is not an absolute effect, but is manifested merely by a decrease in the rate of inactivation by no more than 60% at the most. Thus, one is not justified in invoking a direct relationship with lysine residues because other possibilities may be visualized for an enzyme composed of four subunits in which interaction between substrates and activators has been observed (59), as well as effects of the latter upon protein conformation (25). The phenomenon noted here, therefore, may be a non-specific type of protection of an enzyme by its substrate,

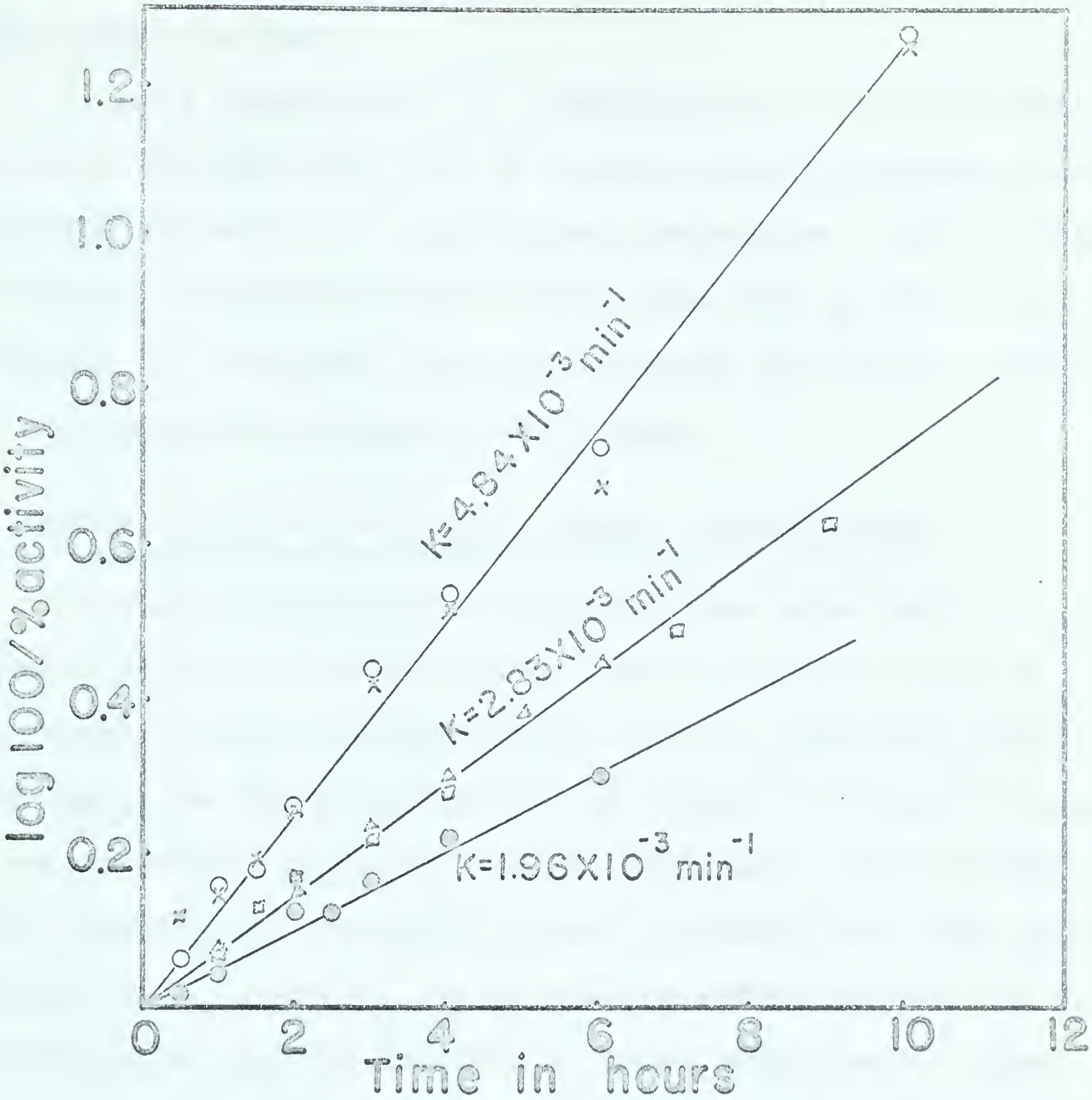


Figure 15. The effect of phosphorylase substrates protection on the enzymatic inactivity by 0.1 M KCNO in 0.1 M Na-glycerophosphate - 0.0015 M Versene buffer, pH 6.8. Phosphorylase a 4 mg/ml .

- ● phosphorylase a preincubated with 0.001 M AMP and 0.016 M G-1-P.
- □ phosphorylase a preincubated with 0.001 M AMP only.
- △ phosphorylase a preincubated with 0.016 M G-1-P only.
- x phosphorylase a preincubated with 1% glycogen.
- o phosphorylase a without substrate preincubation.

such as is commonly observed in connection with such denaturing agents as heat.

In this connection it is interesting to note that AMP and G-1-P decrease the rate of inactivation of phosphorylase a by PCMB (Tovell, D., unpublished observation, 1964). Here, of course, sulfhydryl groups rather than the ε-amino group of lysine are involved, but gross changes in protein structure are observed to occur in both cases.

3. Changes in K_m's and V_{max}'s during carbamylation

If the inactivation of phosphorylase were due to a specific blocking of the active sites or binding sites on the protein, then one would expect this to manifest itself by a change in the Michaelis-Menten kinetics during the course of inactivation unless the inactivation was a true "all-or-none" phenomenon. In either event, it seemed that the determination of K_m's and V_{max}'s at various stages during the inactivation by cyanate might be a useful experiment. Phosphorylase a or b was inactivated with 0.1 M KCNO as described in Methods. Aliquots were withdrawn at intervals and mixed with excess cysteine to stop the inactivation. Enzymic activities were then determined at various concentrations of AMP or G-1-P for phosphorylase b, or G-1-P only for phosphorylase a. The data were analysed by the reciprocal plot method of Lineweaver and Burk and the results are shown in Table II.

To consider phosphorylase a first, the decrease in the V_{max} for G-1-P with increasing time of carbamylation follows a first order type of kinetics. The K_m for G-1-P does not

TABLE II

K_m's and V_{max}'s of G-1-P and AMP for phosphorylase _a_ and _b_ at various carbamylation times

Hours of exposure to 0.1 _M_ KCNO	Phosphorylase _b_				Phosphorylase _a_	
	V_{max} of AMP*	K_m of AMP _M_ x 10^{-5}	V_{max} of G-1-P*	K_m of G-1-P _M_ x 10^{-3}	V_{max} of G-1-P*	K_m of G-1-P _M_ x 10^{-3}
0	9.7	3.2	14.7	3.3	5.88	2.2
1	6.4	3.9	9.25	6.4	5.5	2.3
3	2.8	6.3	5.5	13.0	3.42	2.4
5	1.55	6.78	2.82	13.1	2.02	3.3
7	0.79	6.25	-	-	1.65	8.1
0 (control)	-	-	13.3	3.6	6.3	2.3
5 (control)	11.3	2.1	14.7	4.5	-	-

* Micrograms of inorganic phosphate per minute.

AMP concentrations in reactant mixture were 0.4, 0.6, 1.0, 2.0 and 10.0 x 10^{-4} _M_ at 1.6 x 10^{-2} _M_ G-1-P.

G-1-P concentrations in reactant mixture were 0.4, 0.8, 1.6 and 2.4 x 10^{-2} _M_ at 10 x 10^{-4} _M_ AMP.

show any significant change over the greater part of the inactivation. Not until some 75% of the activity has been lost does the K_m show a marked change.

The decrease in the V_{max} values for phosphorylase b also follows first order kinetics but the change in the K_m values is more difficult to interpret. Certainly an increase in the K_m of G-1-P from 3.3 to 13 millimolar cannot be dismissed as insignificant. The change in the K_m for AMP is less dramatic and could be the result of the change in the G-1-P binding, since these two compounds have reciprocal effects on each other's K_m's (59). There is the possibility, then, that during the inactivation of phosphorylase b by cyanate, various intermediate species of protein appear which have reduced ability to bind substrate or activator but which still retain some catalytic properties. This interpretation is at variance with the results obtained from the kinetics of inactivation and cyanate incorporation. There would also appear to be a distinct difference between the behavior of the two phosphorylases in this respect.

F. Physical Characteristics of Carbamylated Phosphorylases

1. Ultracentrifugal sedimentation behavior

Madsen and Cori (16) discovered in 1955 that phosphorylases a and b react with PCMB resulting in a loss of enzymatic activity and dissociation into subunits of $S^{o}_{20,w} = 5.6S$ or molecular weight 125,000. Wang and Graves (25) also found that at the high ionic strength of 2.5 M NaCl, phosphorylase a breaks into the dimeric form of $S^{o}_{20,w} = 8.3S$. Both of these dissociations were reversible by removing PCMB or NaCl and the enzymatic activity was regained by this process. Thus it was decided to analyze cyanate inactivated phosphorylase in the analytical ultracentrifuge.

The experiments were carried out on both carbamylated phosphorylase a and b. These enzymes had been crystallized six times with a charcoal treatment and passed through Sephadex G-25 gel, then reacted with 0.1 M KCNO in 0.1 M glycerophosphate - 0.0015 M Versene buffer, pH 6.8, at 30°C until completely inactivated. To obtain a partially inactivated enzyme, the carbamylation reaction was stopped by the addition of freshly neutralized cystein buffer solution, pH 6.8. The buffer system used in ultracentrifugal analysis was made up to 1% KCl, 1% glycerophosphate and 0.06% Versene, pH 6.8. The enzyme solution was ultracentrifuged in the Spinco apparatus with a single sector cell at 59,780 r.p.m. (250,000 x g) and temperature was set at $20^{o} \pm 2^{o}C$. The choice of buffer and temperature was based on the greater solubility and stability of the enzymes. When such analyses were carried out, it was found that a new molecular species with a

smaller $S_{20,w}$ was revealed on the sedimentation pattern. In the case of completely inactivated carbamylated phosphorylase b, the new molecular species is a monomeric subunit $S'_{20,w}$ = 5.6S (salt and temperature corrected) as calculated from its sedimentation constant $S_{20,w}$ while about 20-25% remained as the dimeric form $S_{20,w}$ = 8.7S (salt and temperature corrected). In the case of the 60% inactivated phosphorylase b, the monomeric form was only 11% and the dimeric form was 89% (Figure 16). The percentages of the monomer and dimer were determined from area analyses of the sedimentation pattern. It was shown that the rate of inactivation is much faster than the rate of formation of the monomeric form, thus it could be concluded that the enzymatic inactivation is not due to molecular dissociation, but rather that the dissociation is a secondary factor. It was attempted to reassociate the monomeric form to the dimeric form and regain enzymatic activity by dialysis of the carbamylated phosphorylase b against cysteine buffer, pH 6.8, in a cold room for 15 hours, but this procedure was unsuccessful. When carbamylated phosphorylase a was studied in the ultracentrifuge with the same treatment and conditions, it was found that 56% inactivated carbamylated phosphorylase a has no monomeric component, but only tetramer and dimer forms, and their distribution is 69% of $S_{20,w}$ = 8.4S (corrected for salt and temperature) and 31% of $S'_{20,w}$ = 12.7S (corrected for salt and temperature). In completely inactivated phosphorylase a, no tetramer component appeared, but only the dimer and monomer forms, which were 10% of $S_{20,w}$ = 5.86S (cor-

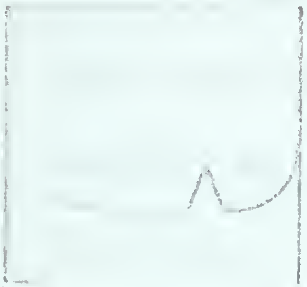
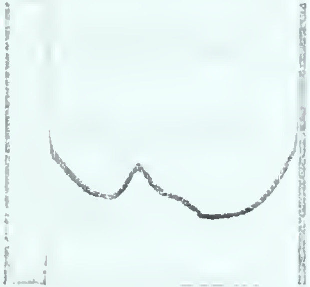
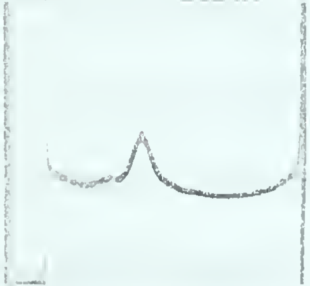
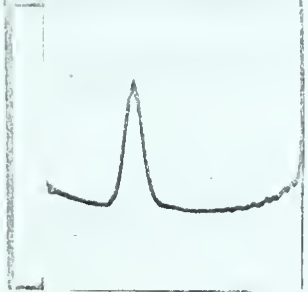
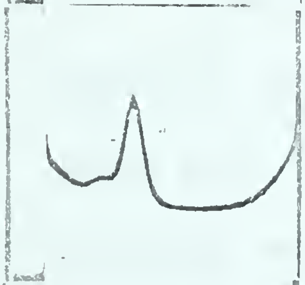
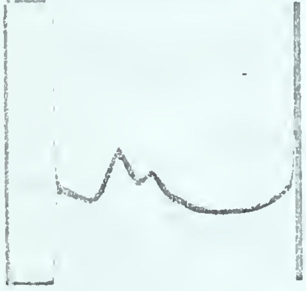
32 minute picture	type of enzyme	enzyme activity	tetramer	dimer	monomer
A 	ph-or-a	100	100% (13.7S=90%) (17.7S=10%	0%	0%
B 	carbamylated ph-or-a	44%	31% (12.7S)	69% (8.4S)	0%
C 	carbamylated ph-or-a	0%	0%	90% (8.4S)	10% (5.86S)
D 	ph-or-b	100	0%	100% (8.65S)	0%
E 	carbamylated ph-or-b	40%	0%	89% (8.4S)	11% (5.36S)
F 	carbamylated ph-or-b	0%	0%	25% (8.7S)	75% (5.6S)

Figure 16. Sedimentation patterns of different types of phosphorylase. (A) 5th recrystallized phosphorylase a, (B) 56% carbamylated phosphorylase a, (C) 100% carbamylated phosphorylase a, (D) 6th recrystallized phosphorylase b, (E) 60% carbamylated phosphorylase b, (F) 100% carbamylated phosphorylase b, in 1% KCl-1% Na-glycerophosphate-0.06% Versene buffer, pH 6.8. Centrifugations were carried out in the Spinco ultracentrifuge operating at a speed of 59,780 r.p.m. Temperature remained 20 ± 2°C.

rected for temperature and salt) and 90% of $S'_{20,w} = 8.4S$ (temperature and salt corrected) (Figure 16). It may be noted that the cyanate induced dissociation of phosphorylase *a* was separated into two steps. The first was the dissociation of tetrameric form into the dimeric form, and the second step involved further dissociation of the dimer to the monomer. It seems that the regular stepwise dissociation reflects that the binding forces stabilizing the quaternary structure of phosphorylase *a* are of two different types. The first weaker force binds the two dimers to form the tetramer, and this can be broken by 2.5 *M* NaCl solution (25). The second, more powerful force connects the two monomer subunits to form a phosphorylase *b* type structure.

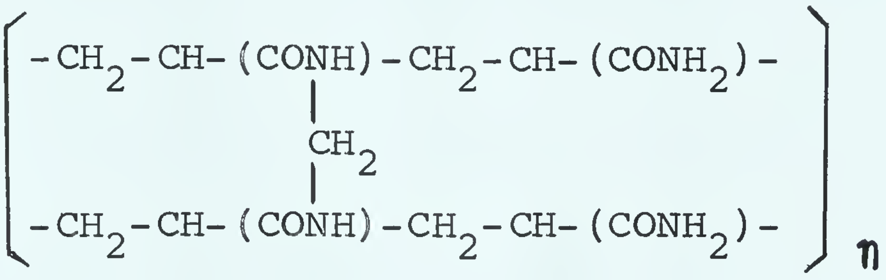
2. Disc electrophoresis

As shown by the ultracentrifugal analyses, the completely inactivated carbamylated phosphorylases *a* and *b* were partly dissociated to monomeric form. An attempt at further separation and identification of these modified phosphorylases *a* and *b* was made by zone electrophoresis.

Starch gel electrophoresis was employed for separating these modified phosphorylases, but these attempts were in vain because starch is a substrate of phosphorylase and it appears that the carbamylated phosphorylase still maintains its affinity for binding starch and agar. Even at the high power of 1,000 volts the carbamylated phosphorylase did not migrate at all. Agar gel electrophoresis was also unsuccessful.

Finally, polyacrylamide gel was used for this purpose.

This gel is made from two organic monomers; acrylamide and N-N' methylene bisacrylamide and ammonium persulfate was used as the catalyst. The chemical component of this penetrable gel has a structure as follows:



$$\left[\begin{array}{c} \mathrm{-CH_2-CH-(CONH)-CH_2-CH-(CONH_2)-} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{-CH_2-CH-(CONH)-CH_2-CH-(CONH_2)-} \end{array}\right]_n$$

The pore size can be adjusted by the concentration of acrylamide. In this experiment, 5% polyacrylamide gel was found most suitable. After staining with amido black and destaining with 7% acetic acid (35), the enzyme bands were a blue color, as shown in Figure 17. Both carbamylated phosphorylases *a* and *b* have nine bands which have coinciding migration distances with the distribution of *3.3* *2.35* *1.7* *1.25* *0.9* *0.65* *0.5* *0.35* and *0.2* cm, respectively, from the original position. Phosphorylase *b* has one band of *2.5* cm migration from the original and phosphorylase *a* has one main band and two tiny bands behind it with migration distances of *2.35* cm. The phosphorylase *a* and *b* mixture has the same migration as phosphorylase *a* except that the main band was wider than phosphorylase *a* alone. Under these conditions separation of phosphorylase *b* from *a* is difficult. The two main factors in the electrophoretic migration distance of a protein molecule are its size and charge. In this case, the size and charge of phosphorylase *b* result in a migration distance similar to that of phosphorylase *a*.

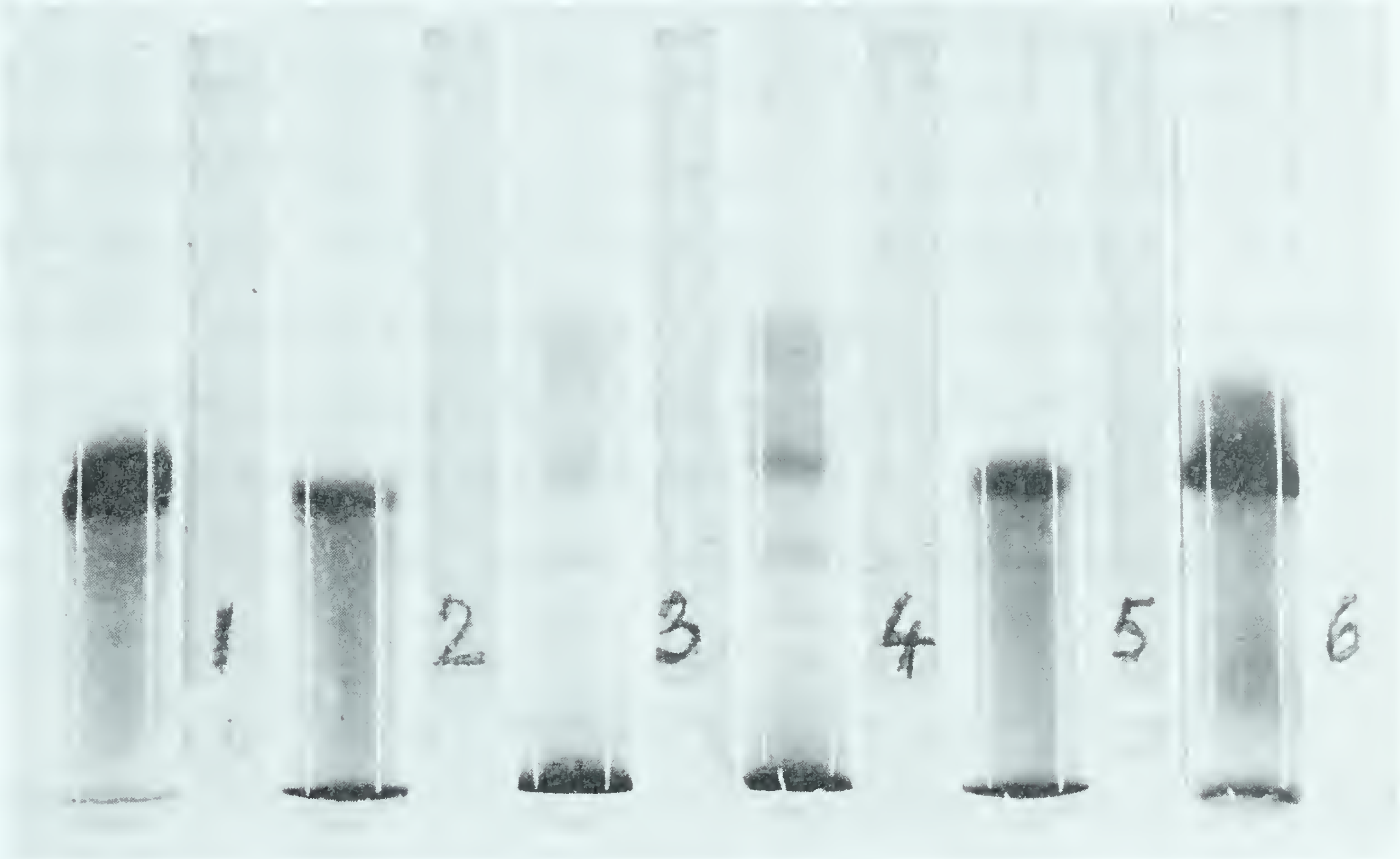


Figure 17. Comparison of disc electrophoresis patterns of phosphorylases *a* and *b*, PCMB treated phosphorylase *b*, the modified phosphorylases *a* and *b* by 0.1 *M* KCNO and liver phosphorylase.

1. phosphorylase *a*
2. liver phosphorylase
3. completely carbamylated phosphorylase *b*
4. completely carbamylated phosphorylase *a*
5. PCMB treated phosphorylase *b*
6. phosphorylase *b*

Experimental conditions are mentioned in Methods.

In the ultracentrifugal technique, the sedimentation patterns were determined mainly by size and molecular weight, and showed that the completely carbamylated phosphorylase a and b were mainly in the dissociated forms of $S^{o}_{20,w} = 8.3S$ and 5.6S, respectively. Both, however, showed nine bands by disc electrophoresis. It can be postulated that the nine bands were the results of differently charged forms. The more fully carbamylated protein molecules would have a greater net negative charge and would be expected to migrate a greater distance toward the anode. It is therefore puzzling that 7 of the 9 bands showed less mobility than the original native enzymes.

PCMB modified phosphorylases a and b have been demonstrated as single peaks in ultracentrifugal analyses (16) and have values of $S^{o}_{20,w} = 5.6S$. When PCMB treated phosphorylases a and b were analyzed by disc electrophoresis technique, they also showed single bands. This confirmed that PCMB modified phosphorylases a and b are homogeneous components. Again this experiment reflected the different behavior of PCMB and KCNO modified phosphorylases.

IV. DISCUSSION

In considering the cause of the inactivation of phosphorylase by cyanate, it was necessary to determine as authoritatively as possible which amino acid residues were actually reacting with the reagent. The evidence presented in this thesis is considered to exclude sulfhydryl groups from this category, in spite of the pronounced reactivity of the sulfhydryl groups of simple organic compounds toward cyanate. Similarly, there does not appear to be a displacement of the pyridoxal-5'-phosphate from the protein. Together with the evidence from amino acid analyses of carbamylated phosphorylase that lysine residues have been converted into homocitrulline residues, one may conclude that the various effects of cyanate upon phosphorylase are due primarily to the carbamylation of the ε-amino groups of lysine.

The dissociation of the tetrameric phosphorylase a into a dimeric form upon the carbamylation of lysine residues is of interest with regard to the nature of the forces holding the four subunits of phosphorylase a together. Earlier work by E.G. Krebs, E.H. Fischer and their associates demonstrated that when two molecules of phosphate are esterified to two specific serine residues of the phosphorylase b molecule, there is a dimerization of such phosphorylated molecules to form phosphorylase a. One might speculate that an ionic interaction between the negatively charged phosphate groups and certain positively charged ε-amino groups is involved in the maintenance of phosphorylase a in its tetrameric form. The observation of Wang and Graves (25) that 2.5 M NaCl also

causes phosphorylase a to dissociate is of significance in this regard. The latter group also suggests(56) that phosphorylase a dissociates spontaneously to a dimeric form, of greater enzymic activity, when it is diluted to very low protein concentrations. These various agents all result in the dissociation of phosphorylase a into two, whereas the blocking of some 18 sulfhydryl groups with PCMB or other reagents results in a dissociation into four subunits of 125,000 molecular weight. It is apparent, then, that the forces holding each pair of subunits together in a dimer differ from those holding two dimers together in a tetramer, and that the first forces are stronger than the second. Phosphorylase fits very precisely the description of an oligomeric protein given by Monod, Wyman and Changeaux (J. Mol. Biol., 1965, in press) in which there is an "isologous" association between two protomers to form a dimer, and a subsequent association between two dimers to form a tetramer. The latter association utilizes different binding sites than those used in the first association to a dimer stage. This model requires that all the protomers be identical. This requirement is probably fulfilled for phosphorylase since the subunit formed as a result of the reaction with PCMB appears to be homogeneous when examined in the ultracentrifuge (16), by free boundary electrophoresis (Madsen, unpublished data), and by disc electrophoresis.

The experiments with phosphorylase a tend to support the idea that the reaction of cyanate with this enzyme is an "all-or-none" process, that is, that when a single protein molecule starts to react with cyanate, all of its potentially available lysine residues react quickly and the protein molecule becomes

completely carbamylated. There would thus be two types of protein molecules present at any one time, those which are completely carbamylated and those which did not react at all with cyanate. Since the completely carbamylated molecules have a different molecular weight than the native protein, it should be possible to separate a partially inactivated enzyme into two types of protein: a completely inactive dimeric form which has incorporated cyanate and a completely active tetrameric form exhibiting no homocitrulline. While this definitive experiment has not yet been performed, the experiments recorded here predict this result.

Thus, partially inactivated phosphorylase _a_ does show a mixture of dimeric and tetrameric forms while completely inactivated enzyme shows a complete dissociation to the dimeric stage. The K_m of G-1-P for phosphorylase _a_ did not show any significant alteration during most of the inactivation process. Some 52 lysine residues per mole of protein apparently behave identically toward cyanate from a kinetic point of view, and also would appear to be of equal importance with respect to enzymic activity. The latter result is hard to reconcile with any direct involvement of these groups with enzymic active centers since there are probably four of these per molecule (16). It is more likely that when the first lysine residue(s) on a protein molecule are carbamylated, the carbamylation of the remainder is potentiated, and 52 react, and an inactive enzyme results.

The actual mechanism by which the carbamylation of

these lysines in phosphorylase a causes a loss of catalytic properties is not immediately apparent from the experiments reported here. The dissociation of the protein to a dimeric form does not provide any answer because phosphorylase b, which is fully active, is a dimer. While glucose-1-phosphate and AMP slow the rate of inactivation, the K_m of the former is not altered for the greater part of the reaction. It has been shown that the carbamylated, inactive enzyme does not bind AMP. These results leave one to state rather nebulously that minor conformational changes, resulting from the carbamylation, may have caused a disruption of the normal configuration of the active centers. It might be noted that the cause of the inactivation of phosphorylase by PCMB, in which the sulfhydryl groups are involved, has also not been satisfactorily explained (16).

The effect of cyanate on phosphorylase b differs from that on phosphorylase a in several respects. There is here a progressive change in the K_m values for AMP and G-1-P during the course of inactivation, and there is a strong tendency for the protein to dissociate to the monomeric stage. Further experimentation will be necessary to resolve these differences.

The results of the disc electrophoresis experiments argue against the "all-or-none" hypothesis since a pronounced heterogeneity was demonstrated. As was discussed on page 29, it is possible that the lysine residues attacked by isocyanate are all equivalent and that the carbamylation of each leads to a small change in physical structure and activity.

V. SUMMARY

Both phosphorylases a and b have been found to be inactivated by potassium cyanate. The course of the inactivation follows pseudo-first order kinetics. The rate of inactivation varies directly with the concentration of cyanate but the variation of rate is less than first order with respect to protein concentration. The rates of incorporation of carbon-14 labelled cyanate into both phosphorylases a and b parallel the rates of inactivation of the two enzymes. Identical first order constants for the rates of incorporation and inactivation are obtained for each protein if it is assumed that 52 residues per molecule of a and 23 per molecule of b must be carbamylated to yield 100% inactivation.

The addition of an excess of cysteine stops further inactivation by cyanate at any point during the reaction but does not produce any reactivation. The cause of the inactivation of phosphorylase by cyanate does not appear to be the carbamylation of sulfhydryl groups because the same number of sulfhydryl groups in the carbamylated as the native protein can be titrated with PCMB. Furthermore, the rate of incorporation of labelled cyanate into phosphorylase a or b in which the sulfhydryl groups have been blocked with PCMB is the same as the rate of incorporation into the native proteins.

The inactivation of phosphorylase by cyanate does not appear to be a displacement of pyridoxal-5-phosphate from lysine residues because the typical spectrum of the protein-

bound coenzyme is not altered during the carbamylation reaction. While some 40 to 50% of the PLP is lost during dialysis of the carbamylated protein, this may be due to a labilization of binding forces in the altered protein. Finally, phosphorylase which has been treated with sodium borohydride, so that the linkage between PLP and lysine has been converted to a stable covalent bond, is still readily inactivated with cyanate.

Amino acid analyses of carbamylated phosphorylases a and b indicated that varying proportions of the lysine residues had been converted to homocitrulline. No other marked changes in the amino acid composition could be discerned with this technique. In conjunction with the experiments summarized above, this data agrees with the hypothesis that the effects of cyanate on phosphorylases are due solely to the carbamylation of lysine residues. It may be noted that treatment of either phosphorylase with cyanate in 8 M urea did not result in the conversion of more than 50% of the lysine residues to homocitrulline.

Carbamylated phosphorylase a does not cause the spectral shift in AMP which is caused by the native enzyme and this might be interpreted as a difference in binding of the nucleotide, either quantitative or qualitative. Both AMP and glucose-1-phosphate decrease the rate of inactivation by cyanate, and this effect is addative when both are present together. Glycogen has no effect. The K_m of G-1-P for phosphorylase a did not change significantly through the greater part of the inactivation process. On the other

hand, the K_m's of AMP and G-1-P did increase during the inactivation of phosphorylase b by cyanate.

Ultracentrifugal analyses of carbamylated phosphorylase a suggest that the completely inactivated enzyme exists in a dimeric form rather than the usual tetrameric form. Partially inactivated phosphorylase a shows a partial conversion to the dimeric form, although a strictly parallel relationship between inactivation and dissociation could not be demonstrated. Nevertheless, there was some evidence for an "all-or-none" type of reaction between phosphorylase a and cyanate, as suggested by the kinetic analyses discussed above. Partially inactivated phosphorylase b existed primarily in its usual dimeric form, but upon complete inactivation some 75% appeared as the monomeric form. In contrast, completely inactivated phosphorylase a showed only a trace of the monomeric form.

Disc electrophoresis of carbamylated phosphorylases a and b showed some nine bands in each case, whereas the native proteins, as well as the monomer produced by treatment with PCMB, show only single bands.

VI. BIBLIOGRAPHY

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